New Myelospecific Selfinactivating Gammaretroviral Vectors for Gene Therapy of p47^{phox} Deficient Form of Chronic Granulomatous Disease

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Zusammenfassung

Die septische Granulomatose (chronic granulomatous disease: CGD) umfasst eine Gruppe von angeborenen Immundefekten, die durch einen Ausfall des oxidativen Burst gekennzeichnet sind. Der fehlende Burst beeinträchtigt die antimikrobielle Aktivität der Phagozyten.

60% aller CGD Patienten leiden an Mutationen des *cybb* Gens, welches auf dem X-Chromosom vorliegt (X-CGD). 30% weisen eine Mutation des *ncf1* Gens auf, welches das $p47^{phox}$ Protein kodiert. In einer kürzlich durchgeführten gamma (γ)-retroviralen Gentherapie (GT) Studie zur Behandlung von X-CGD konnten erste klinische Erfolge erzielt werden. Diese Therapieform ist eine Alternative zur Knochenmarktransplantation, die bei fehlendem HLA-identischen Spender häufig mit hoher Morbidität, Sterblichkeit und Transplantatversagen verbunden ist. Jedoch waren die retroviralen Gentherapie-Studien für X-CGD und ADA-SCID von klonaler Dominanz oder leukämischem Verlauf als Langzeitfolge von Transaktivierungsvorkomnissen begleitet.

Eine Möglichkeit, die Risiken des Auftretens von Transaktivierung zu vermindern, ist der Gebrauch von γ-retroviralen selbst-inaktivierenden (SIN) Vektoren, bei denen die viralen Elemente (Promotoren- und Enhancersequenzen) während der Integration inaktiviert werden. Die Expression des Transgens wird von einem internen gewebsspezifischen Promotor angetrieben, der im Idealfall selbst keine Enhancer Aktivitäten besitzt und die Expression des Transgens auf terminal differenzierte Granulozyten/Monozyten begrenzt.

Für die $p47^{phox}$-defiziente Form von CGD ist die Knochenmarktransplantation die momentan einzig verfügbare Therapie. Deshalb liegt der Fokus dieser Doktorarbeit in der Entwicklung eines sicheren γ-retroviralen SIN GT Vektors, um den $p47^{phox}$ CGD Phänotyp zu korrigieren.

Im ersten Teil der Doktorarbeit klonierten wir unterschiedliche myelospezifische Promotorkandidaten in γ-retrovirale SIN Vektoren, die die $p47^{phox}$ Transgenexpression antreiben. Die Konstrukte wurden auf myelospezifische Expression und funktionelle Wiederherstellung des oxidativen Burst in transduzierten hämatopoietischen Stammzellen überprüft, welche in der Zellkultur weiter zu Granulozyten ausdifferenziert wurden. In diesem Auswahlverfahren haben wir einen neuen erfolgsversprechenden myelospezifischen microRNA-223 (miRNA-223) Promotor gefunden, der die $p47^{phox}$ Expression auf Granulozyten begrenzte. Im Weiteren führte die miRNA-223 kontrollierte Expression zur funktionellen Wiederherstellung der NADPH-Oxidase in kultivierten Zellen.

Im nächsten Schritt wurde dieser myelospezifische miRNA-223 Promotor in einer GT bei $p47^{phox}$ -/- Mäusen verwendet, und das Ergebnis wurde mit einem konstitutiv aktiven Kontrollvektor verglichen. Die GT mit diesem miRNA-223 Vektor zeigte eine klare myelospezifische $p47^{phox}$ Transgenexpression sowie eine intakte funktionelle NADPH-Oxidase in den Zielzellen (Neutrophile) *in vivo*. Die beschriebene Kombination eines γ-

retroviralen SIN Vektors mit einem strikten myelospezifischen Promotor zeigt bei der GT for CGD eine deutliche Verbesserung der Sicherheit der GT für CGD.

Zu guter Letzt ist der Nachweis der $p47^{phox}$ Expression eine zwingende Anforderung für das Monitoring einer GT. Die intrazelluläre Immunfärbung des zytoplasmatischen $p47^{phox}$ Transgenprodukts interagiert allerdings mit der Analyse der Aktivität des oxidativen Burst. Um dieses Problem zu lösen, entwickelten wir erfolgreich ein neues Reportersystem. In diesem System wird das zytoplasmatische $p47^{phox}$ über die Koexpression eines zellulären Oberflächenmarker gemessen, der in einem 1:1 molaren Verhältnis exprimiert wird.

Summary

Chronic granulomatous disease (CGD) comprises a group of primary immunodeficiencies characterized by the failure of respiratory burst and thereby an impaired antimicrobial activity of phagocytes. 60% of all CGD patients suffer from mutations in the X-linked *cybb* gene (X-CGD), followed by 30% with a mutation in the *ncf1* gene encoding $p47^{phox}$. First clinical success could be achieved in a recent gamma (γ)-retroviral X-CGD gene therapy (GT) study. This GT represents a therapy complementary to bone marrow transplantation (BMT), which is associated with unacceptable high rates of morbidity, mortality and graft failure, except in very selected cases in which a HLA-identical donor is available. However, retroviral GTs for X-CGD and ADA-SCID were accompanied with clonal dominance or leucemic progression as long-term effects of transactivation events.

One option to minimize the risks for transactivation in CGD GT is the use of γ-retroviral self-inactivating (SIN) vectors, in which the viral promoter/enhancer elements are inactivated upon integration. The transgene expression is driven by an internal tissue-specific promoter element which ideally lacks enhancer activity and restricts transgene expression to terminally differentiated granulocytes/monocytes.

For the $p47^{phox}$-deficient form of CGD, BMT is the only therapy available at present. Therefore, the thesis focused on the development of a safer γ-retroviral SIN vector for gene replacement therapy to correct the $p47^{phox}$ CGD phenotype.

In the first part of this thesis we cloned various myelospecific promoter candidates into γ-retroviral SIN vectors driving $p47^{phox}$ expression. The constructs were screened for myelospecific expression and functional reconstitution of the NADPH oxidase in transduced murine $p47^{phox}$ -/- hematopoietic stem cells upon propagation to granulocytes in cell culture. By this screen we have identified a new potent myelospecific promoter, microRNA-223 (miRNA-223), which results in $p47^{phox}$ expression exclusively in granulocytes. Furthermore, miRNA-223 controlled $p47^{phox}$ expression resulted in functional reconstitution of the NADPH-oxidase in cell culture.

Next, this myelospecific miRNA-223 promoter was utilized in an *in vivo* GT in $p47^{phox}$ -/- mice and the outcome was compared to the constitutive active control vector. GT with miRNA-223 vector resulted in a clearly myelospecific $p47^{phox}$ transgene expression and restored the function of the NADPH oxidase in target neutrophils *in vivo*. The presented combination of a γ-retroviral SIN vector with a strictly myelospecific promoter represents a major safety improvement in CGD GT.

Finally, the detection of $p47^{phox}$ expression is a mandatory requirement for GT monitoring. Intracellular immunostaining of the cytoplasmic $p47^{phox}$ transgene product interferes, however, with the analysis of respiratory burst activity. To overcome this limitation we

successfully developed a new reporter system for measuring cytoplasmic $p47^{phox}$ by co-expression of a cellular surface marker in a molar ratio of 1:1.

General Introduction

Chapter Overview

The central issue of this work was to develop a safe retroviral-mediated gene therapy (GT) for the $p47^{phox}$ deficient form of Chronic Granulomatous Disease (CGD). Therefore, the first part focuses on the description of CGD. Underlying mechanisms causing CGD, the main clinical features and currently available treatment options for CGD, especially GT, are described including the risks of current retroviral GT protocols. The second part summarizes basics about the biology of hematopoietic stem cells (HSC) and hematopoiesis, since HSC are the prime target organ of CGD GT. The last part illustrates basic features of retroviruses and their use as vehicles for gene transfer. Furthermore, a viral system for co-expression of two proteins is introduced.

CGD

General description of CGD

CGD is a rare primary inherited immunodeficiency leading to severe and life-threatening bacterial and fungal infections. CGD is normally diagnosed in infancy and has an incidence of between 1:200000 and 1:250000 live births[1]. The disease is caused by deletion/mutations in any one of the four gene encoding the subunits of the superoxide-generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in phagocytic cells (neutrophils, monocytes, macrophages and eosinophils). The NADPH oxidase is elementary for microbial killing by reducing molecular oxygen to superoxide, which subsequently reacts to form secondary reactive oxygen species (ROS)[2]. Thus phagocytic cells from CGD patients with absent or malfunctioning NADPH oxidase subunits are able to phagocytose pathogens but are unable to kill them (figure 1). As a result, affected individuals suffer from recurrent life-threatening bacterial and fungal infections. Abnormally exuberant inflammatory responses of CGD patients can lead to formation of inflammatory granulomas to which CGD owes its name[1, 3].

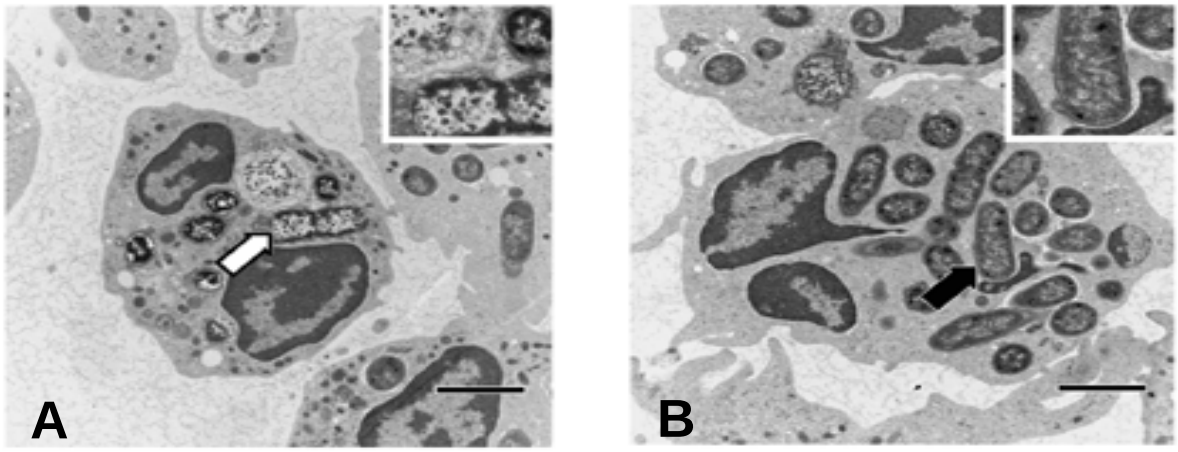

Figure 1 Transmission electron microscopy of opsonized *Escherichia coli* (*E.coli*) strain ML-35 2.5 h after phagocytosis by healthy control (**A**) or CGD (**B**) granulocytes. Black arrow denote undigested *E. coli* inside the phagocytic vacuole. White arrow indicate *E. coli* degradation. Insert on the right upper corner shows magnifications of digested (**A**) and undigested (**B**) bacteria. Scale bars 2 μm (modified from[4]).

The NADPH oxidase: Function and properties

NADPH oxidase is found in professional phagocytes (neutrophils[5], eosinophils, monocytes and macrophages[6]). It is a phagosomal and plasma membrane-associated enzyme complex. The structure of the NADPH oxidase is complex (figure 2) consisting of two membrane-bound elements (gp91^{phox} and p22^{phox}) and three cytosolic components (p67^{phox}, p47^{phox} and p40^{phox})[7, 8]. The names of the oxidase subunits are referred to their apparent molecular mass (kDa) and have been given the signation *phox*, for *ph*agocyte *ox*idases[9]. In table 1 properties of the phox components are summarized. In addition, the low-molecular weight GTP-binding protein (either Rac 1 or Rac 2) associates with and is involved in the regulation of the NADPH oxidase[10, 11].

The multicomponent enzyme harbours a redox center that transfers electrons from cytoplasmic NADPH onto extracellular (or intraphagosomal) molecular oxygen thereby generating superoxide according to the following reaction[7, 12]:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

The electron transfer from NADPH to oxygen is a multistep process. gp91^{phox} and p22^{phox} form a membranspanning flavo-hemeprotein, called flavocytochrome b558, containing two molecules of heme and one molecule of flavin adenin dinucleotide (FAD) through which the electrons are transported sequentially[8]. NADPH cannot bind to flavocytochrome b558 unless the complete enzyme has been assembled during activation, and only then electron transfer actually takes place (figure 2b).

The soluble regulatory components p47^{phox}, p67^{phox} and p40^{phox} are found within the cytosol in resting cells[9, 13]. Phagocyte activation leads to phosphorylation of cytosolic factors (p47^{phox},

p67^{phox}, p40^{phox}, Rac) and to their translocation to the membrane. There, p47^{phox} and p67^{phox} bind to flavocytochrome b558 and build up the activated NADPH oxidase complex.

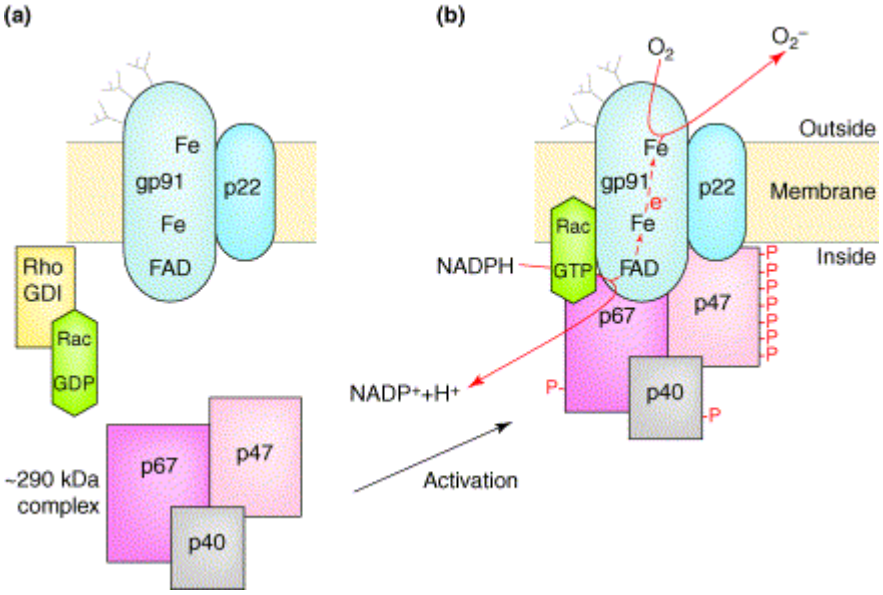

Figure 2 A model of phagocyte NADPH oxidase ('respiratory burst') activation. (a) In its resting state the oxidase is composed of membrane-bound and cytosolic protein components. The integral membrane proteins gp91phox and p22phox together form the flavocytochrome b558, with the larger subunit containing the FAD and heme- (Fe) binding sites. The cytosolic components include p40^{phox}, p47^{phox} and p67^{phox}, which exist together in a 290 kDa complex. In its inactive GDP-bound state, the small GTP-binding protein Rac is also cytosolic and is bound to Rho GDP-dissociation inhibitor (RhoGDI). (b) Upon activation p47^{phox}, p67^{phox} and p40^{phox} become stably associated with the plasma membrane primarily through interactions between p47^{phox} and the subunits of flavocytochrome b558. This translocation process is accompanied by, and perhaps requires both the release of Rac from RhoGDI, its conversion to an active (GTP-bound) state and its association with the plasma membrane, and the phosphorylation of p47^{phox} (at multiple sites), p40^{phox} and p67^{phox}. In a manner that is not yet fully understood, binding of the cytosolic components activates the flavocytochrome to catalyze the transfer of electrons from NADPH to oxygen, via the flavin and heme redox centers in gp91phox, to form $O2^-$ (red dashed line). The compartment labeled 'inside' is the cytoplasmic space; 'outside' refers either to the extracellular or phagosomal space (modified from[13]).

	flavocytochrome b558				
	gp91^{phox}	**p22^{phox}**	**p47^{phox}**	**p67^{phox}**	**p40^{phox}**
Gene and locus	*cybb*; Xp21.1	*cyba*; 16q24	*ncf-1*; 7q11.23	*ncf-2*; 1q25	*ncf-4*; 22q13.1
Amino acids	570	195	390	526	339
Molecular weight:					
Predicted	65,338 Da	20,959 Da	44,684 Da	59,735 Da	39,039 kDa
By SDS-PAGE	~90 kDa	22 kDa	47 kDa	67 kDa	40 kDa
Glycosylation	Yes	No	No	No	No
pI	9.26	10.1	9.58	6.12	7.28
Phosphorylation	No	Minor	Yes	Minor	Yes
Location in neutrophil					
Resting	Membrane of specific granule and plasma membrane		Cytosol		
Stimulated	Plasma membrane and phagosomal membrane		Membrane associated		
Abundance pmol/10^6 cells (cytosol conc.)	1.0–2.0		6.0 (2750 nM)	1.0 (460 nM)	1.0 (460 nM)
Functional domains	C-terminus binds cytosolic components. Heme, FAD and NADPH binding regions	C-terminal proline-rich region	Phosphorylation sites PX domain, 2 SH3 domains, proline-rich region	Tetratricopeptide repeat, 2 SH3 domains, proline-rich domains	PX and SH3 domains, octicosapeptide repeat

Table 1 Properties of the phagocyte respiratory burst oxidase (phox) components (modified from[8])

The superoxide produced by electron transfer of NADPH oxidase itself contributes by only 50% to the bactericidal activity[14]. Within the phagosome, superoxide is spontaneously or enzymatically converted into hydrogen peroxide (H_2O_2), which may then react further to toxic compounds such as hydroxyl radicals OH^-, hypochlorous acid catalyzed by myeloperoxidase[2] and peroxynitrite[7] (figure 3).

$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$[12]

Although superoxide derivatives were first believed to be solely responsible for the antimicrobial activity of phagocytes, new insights have revealed additional mechanisms of the NADPH oxidase[2]. The NADPH oxidase induces killing in an indirect way via liberation of microbicidal azurophil granule proteases (cathepsin G and elastase) into the phagosome. Segal et al[15] have seen that mice deficient in the granule proteases cathepsin G and elastase are ineffective to eliminate *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*), even though they had normal NADPH oxidase activity. Moreover, protease inhibitors could impair the ability of human neutrophils to kill *S. aureus*. Based on these results, they proposed a new model for microbial killing in neutrophils[2] (figure 3). The NADPH oxidase drives an electrogenic process by transferring large amounts of electrons into the phagocytic vesicle. The compensation of enzymatic charge separation is still a matter of debate.

Segal and coworkers developed a model in which the charge transfer is partly compensated by the influx of protons (H^+) from the cytosol through the NADPH oxidase[2]. The phagosomal membrane depolarization and maybe a drop of pH just beneath the plasma membrane elevate cytosolic calcium (Ca^{2+}) concentrations which fully open calcium^{2+}-activated K^+ (BK_{ca}) channels[16]. The influx of potassium (K^+) partly elevates the pH in vesicle and most importantly leads to a hypertonic environment.

On the other hand, the latest models claim that charge separation is compensated by voltage-gated proton channels and not by the BK_{ca} channels[17-19].

The change of intraphagosomal pH and ion composition results in the liberation and activation of granule proteases and antimicrobial peptides[2, 15]. The functional role of NADPH oxidase in antimicrobial peptide processing and activity is not clear so far.

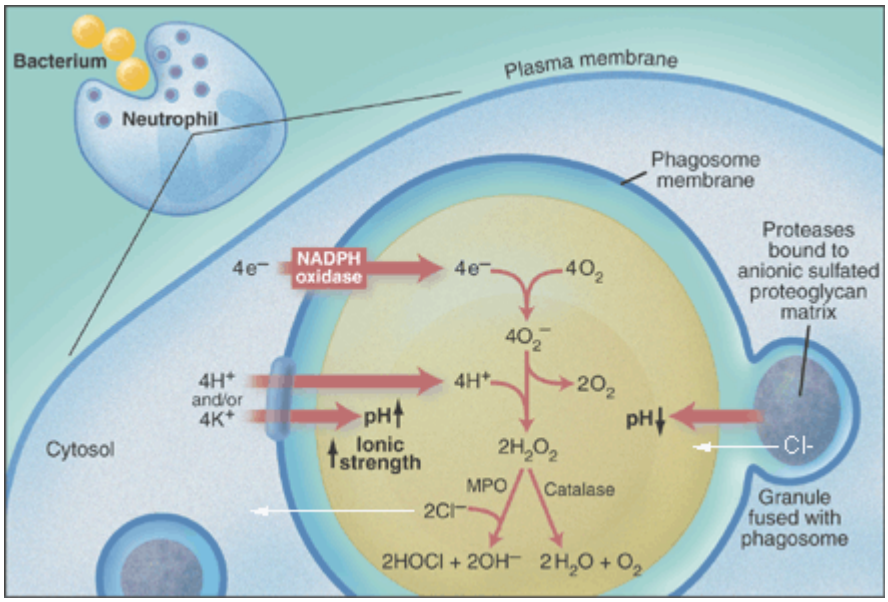

Figure 3 In the "belly" of the phagocyte (according to the model of Segal). Within the small space between an ingested bacterium (shaded area) and the membrane of the phagosome, a number of chemical reactions take place which lead to bacterial killing by neutrophils (see text for detail; modified from[20]).

Since the discovery of the indirect killing mechanisms, the open question still remains, if the oxidative products are directly involved in microbial killing[7, 20]. Segals et al argue[2], superoxide generation is needed only to increase the ionic strength within the phagosome, which then allows solubilization of the proteases and does not contribute directly to the killing process. In others view[9, 20, 21] there is a large body of evidence that oxidants are involved directly in killing. For example, CGD neutrophils are able to kill catalase-negative bacteria (which excrete H_2O_2) and also catalase-positive bacteria, if they coingest glucose oxidase, which generates H_2O_2[22-24]. Recently *ex vivo* differentiated neutrophils from CD34+ stem cells with deficient phagosomal granule development showed significant reduced killing activity against *E. coli* in comparison to peripheral blood neutrophils, but could still kill 50% of *E. coli* [14]. These findings point to a direct contribution of ROS in the killing process.

In addition to active phagocytosis and intracellular killing by antimicrobial peptides and ROS, neutrophil extracellular traps (NET) were found to be involved in extracellular microbial killing[25]. In NET mediated killing, a complex of granule proteases, antimicrobial peptides and chromatin-DNA is supposed to kill trapped bacteria and fungi. NETs are released upon a specialized form of cell death that is NADPH oxidase dependent[26].

Molecular genetics of CGD

More than two-thirds of all CGD cases result from mutations in the X-linked *cybb* gene encoding the gp91^{phox} subunit (X-CGD), followed by autosomal recessive forms of CGD: 30% of all CGD cases are associated with mutations in the *ncf-1* gene encoding p47^{phox}, while only 5% of cases are due to mutations in *cyba* (p22^{phox}), and *ncf-2* (p67^{phox})[1, 13]. A single

patient with heterozygous mutation in the *rac 2* gene was identified[27, 28] with a CGD phenotype. CGD cases due to mutations in *ncf-4* encoding p40^{phox} have not yet been reported[7].

Heyworth and coworkers[13] summarized 410 defects in the four affected genes. Only 19 resulted in a normal level of inactive or weakly active protein. The remaining 95% of CGD mutations resulted in a complete absence or greatly diminished level of protein, either because the affected gene is partially or completely deleted, or because the aberrant protein product or mRNA is unstable.

Mutations in *ncf-1* encoding p47^{phox} cause the most common autosomal recessive form of CGD. Unlike the other variants of the disease, in which a high degree of heterogeneity exists among mutations, a single mutation has been identified in about 94% of affected alleles in patients with p47^{phox} deficiency[13]. This mutation is a base deletion of gt at the beginning of exon 2 that causes a frameshift resulting in a premature stop codon[29]. This gt deletion is the key difference between *ncf-1* and a pair of highly homologous pseudogenes that are physically close to the functional gene. Two studies have shown that the predominance of the gt deletion probably happens during recombination events (crossover) between the functional *ncf-1* and its highly homologous pseudogene[30, 31].

Clinical features of CGD

CGD patients suffer from severe recurrent bacterial and fungal infections of body surfaces, e.g. the skin, the airways, the gut and draining lymph nodes. Through contiguous and haematogenous spread also many internal organs can be affected e.g. the liver and the bones. The major clinical manifestations of CGD are pyoderma, pneumonia, inflammation of the gastrointestinal tract, lymphadenitis, liver abscess and osteomyelitis[1, 3]. Many CGD patients also develop chronic inflammatory granulomas, which are distinctive hallmark of this disorder. Mechanistically the formation of granulomas is believed to reflect a dysregulated inflammatory response and/or inefficient degradation of inflammatory mediators in the absence of respiratory burst-derived oxidants[32].

S. aureus is the pathogen most frequently found in CGD patients. *Aspergillus* species or *Burkholderia cepacia* are the most common cause of death in these patients, contributing towards an overall death rate of 2% (for autosomal CGD) and 5% (for X-CGD)[1] per year. Other pathogens that are frequently found in insolates from CGD patients include *Nocaria* species, and a variety of Gram-negative enteric bacilli such as *Serratia marcescens*, *Salmonella* species.

Mouse models of CGD

Four different CGD mouse models have been characterized: mouse models resembling the gp91^{phox} deficient CGD[33], the p47^{phox} deficient CGD[34], the p22^{phox} deficient CGD[35] and deficient p40^{phox} CGD[36]. CGD mice have abnormalities in both host defense and inflammation which are similar to their human counterpart. CGD mice show also an increase in susceptibility to the opportunistic pathogens, *Burkholderia cepacia* and *Aspergillus* species, two organisms that are particularly problematic in CGD patients. Other organisms, e.g. *S. aureus*, *Salmonella typhimurium*, *Mycobacterium tubercolosis* and *Candida* species show increased virulence in both, CGD mice and in CGD patients[33]. Both gp91^{phox} - and p47^{phox} deficient CGD mice have abnormalities in inflammatory responses[9]. For example gp91^{phox}-/- mice exhibit an exaggerated acute inflammatory response after instillation of sterilized hyphae into the lung or upon intradermal injection, which evolves into a chronic granulomatous infiltrate[32].

Treatment of CGD

Conventional management

CGD patients need to be instructed about their condition and its management, and checked regularly by experienced physicians. They should as far as possible reduce exposure to potentially infectious agents. They require prophylactic antimicrobial therapy which consists of lifelong prophylaxis with antibiotics such as trimethoprim/sulfomethoxazol (cotrimaxole). This antibiotic has a broad activity against Gram-negative bacteria and is concentrated inside host cells[3]. For antifungal prophylaxis the lipophilic itraconazole is taken, because it displays high activity against *Aspergillus* spp. A subgroup of “variant” X-linked CGD patients, who have some residual respiratory burst activity through possessing splice site mutations, can be treated with interferon-gamma (IFNγ), a macrophage-activating cytokine produced by T cells and natural killer cells[37, 38]. IFNγ improves splicing efficiency. Thereby a small amount of normal gp91^{phox} transcript is generated, which leads to an increase in cytochrome b expression and normal killing activity in phagocytes of “variant” CGD patients. Acute infections in CGD patients are treated most successfully by prompt and prolonged therapy with the appropriate antibiotic therapy[3]. Additionally during severe infections the immune system can be temporarily supported by white cell transfusions, which is however, limited by the risk of antibody formation against foreign human leukocyte antigen (HLA). Overall, these prophylactic medications ameliorate the symptoms of the disease, as seen by improved health conditions and survival rates[1].

Cure of the disease: Hematopoietic stem cell transplantation (HSCT)

One option to cure CGD is hematopoietic stem cell transplantation (HSCT). The overall success rate of conventional HSCT (myeloablative marrow conditioning, HLA-identical donor material) is 81%, with an overall mortality of 15%[39]. Survival in these patients without infection at transplantation was excellent. Most cured patients had over 95% circulating donor cells and pre-existing infections and chronic inflammatory lesions have been cleared in all engrafted survivors. Therefore myeloablative marrow conditioning HSCT is a valid therapeutic option for children with CGD and having HLA-identical donor. Unfortunately a highly compatible donor is only available for 52% of CGD patients [40]. The major risk factors associated with HSCT are graft-versus-host disease and inflammatory flare-ups at sites of infection. In the absence of a HLA-identical sibling or unrelated donor, haploidentical HSCT has been performed only twice and is considered rather risky because of delayed immune reconstitution and graft failure[3].

In vitro fertilization (IVF) combined with preimplantation HLA-testing to select an HLA-genoidentical, disease-free sibling embryo as a "savior baby" for successful HSCT is another treatment option[41] (not allowed in Switzerland). Beside legal and ethical concerns this demanding treatment needs a young maternal age and the firm wish of the parents to have another healthy child. Moreover, the probability of successful pregnancy in the most experienced IVF centers is only around 10%.

Stem cell GT

CGD is considered to be a suitable candidate for a GT approach: All genes encoding the subunits of the NADPH oxidase complex are known and are not involved in cell proliferation. Several assays are established to determine superoxide production after GT in order to estimate functional correction of the disease. Furthermore, functional correction of as few as 5% of neutrophils should be sufficient to alleviate the symptoms of the disease based on the experience in X-linked CGD carriers[42]. These facts and observations have motivated to develop a GT protocol for CGD treatment.

Preclinical studies of GT for CGD

For the first time in 1992, retroviral GT restored respiratory burst activity in Epstein-Barr virus (EBV)-transformed peripheral blood CGD B-cells *ex vivo* [43]. Later on, human CGD CD34+ progenitor cells or cells from the human gp91^{phox} knockout cell line (PLB985 X-linked CGD) have been tested for the feasibility of GT. Beside retroviral vectors, an adeno-associated virus was successfully tested as well in immortalized CGD B cells[44]. In all cases respiratory

burst activity has been restored, albeit at different levels depending on the expression vector used in each experiment[45-50].

The availability of animal models for the two main forms of CGD had significant impact on preclinical CGD GT development. Respiratory burst activity was reconstituted in both gp91^{phox} and p47^{phox} mouse models in which the transplanted HSCs were transduced with gp91^{phox} [50-52] or p47^{phox} [53, 54] expressing vectors prior to reinfusion into lethally or sublethally irradiated recipients. Dinauer *et al*[51] reported that 50% to 80% of granulocytes in X-linked CGD mice had a reconstituted superoxide activity and an intact host defense against *Aspergillus fumigatus* after GT. The use of improved retroviral vector backbones, like those derived from spleen focus-forming virus (SFFV) have further enhanced gp91^{phox} expression in hematopoietic cells. Moreover, serial transplantations of transduced cells into secondary and tertiary gp91^{phox} -/- recipients demonstrated even long-term correction of X-linked CGD[55]. The ectopic expression of gp91^{phox}, which is normally only found in myeloid cells, was well tolerated, since transgene expression did not affect engraftment, survival, proliferation or differentiation of transduced cells *in vivo*[50-52, 55, 56]. The clearance of pathogens by gene-transduced cells was checked by challenge experiments. In these experiments animals transplanted with gene-corrected cells or mixtures of CGD and wild-type cells were exposed to bacteria and fungi. It could be shown that the minimal protecting level of superoxide production varies depending on the pathogens used to challenge the animals. For example, for mice challenged with *Burkholderia cepacia* 30% gene-corrected cells were necessary, whereas against *Aspergillus fumigatus* spores only 11% gene-corrected cells were sufficient. Similar observations were made by subcutanous injection of sterilized *Aspergillus fumigatus hyphae* into X-linked CGD mice. These mice developed an acute and chronic inflammation with granuloma formation resembling that observed in CGD patients. Mice with more than 20% gene-corrected cells were protected from granuloma formation, whereas lower transduction levels were still associated with chronic infections[57]. In 1997 the first retroviral GT for p47^{phox}-deficient CGD was performed in p47^{phox} -/- mice[54]. ScaI^{+} p47^{phox}-/- marrow progenitor cells were transduced with a moloney murine leukemia virus (MLV)-based retroviral vector which encoded the human form of p47^{phox}. Transduced progenitor- and stemcells were transplanted into moderately irradiated (500cGy), G-CSF preconditioned p47^{phox} -/- mice. A portion of peripheral blood neutrophils (12% after 4 weeks, 2.6% after 14 weeks) showed restored respiratory burst activity in a dihydrorhodamine 123 (DHR) assay. GT treated p47^{phox} -/- mice showed significantly lower bacteremia levels and higher survival rate after challenge with CGD pathogen *Burkholderia cepacia* compared to untreated animals[53].

All together, these preclinical studies have pointed out, that also low superoxide levels and/or a small fraction of fully gene-corrected cells might already protect CGD patients from severe and life-threatening infections and improve the clinical status and quality-of-life of CGD

patients[51-54, 57, 58]. However, in cases where gene-transduced cells will not produce the same levels of superoxide as observed in wild-type cells, larger amounts of gene-corrected cells (30%) may be required for an effective antimicrobial activity[57].

Clinical gene therapy studies for CGD

In 1995 the first clinical GT trial for $p47^{phox}$ deficient CGD was led by Harry Malech at the National Institutes of Health (NIH)[59]. Five $p47^{phox}$ deficient CGD patients were treated with granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood CD34+ cells after *ex vivo* transduction with a $p47^{phox}$-expressing retroviral vector. A large number of transduced CD34+ cells (total numbers between 2×10^6 and 2×10^8) were reinfused into the patients without prior BM conditioning. The level of functionally corrected granulocytes was low (range between 0.004% and 0.05% of total peripheral blood granulocytes) and persisted at this level for up to 6 months after reinfusion. 3 years later the same research group initiated a second GT study for X-linked CGD. The protocol included several modifications: CD34+ cell mobilization was achieved using Flt3 ligand and G-CSF; retroviral transduction of CD34+ cells was done on four subsequent days, resulting in high rate of colony-forming cells (range between 48% and 89%). Furthermore, patients received transduced CD34+ in two cycles 50 days apart, without BM conditioning. Despite these modifications, the level of cells with restored burst activity was still low (range between 0.2 and 0.6%) and persisted at this level for the following 4 to 6 months[60]. In 2004 a third clinical GT study for X-CGD was performed by Dinauer et al[61], at Indiana University. In study number three, a murine stem cell virus-derived retroviral vector was used for transduction. Again, without prior BM conditioning after reinfusion only 0.1% of peripheral blood neutrophils showed restored respiratory burst activity for almost 9 months.

In summary, the first clinical GTs provided evidence that the GT approach to treat CGD is feasible. Retrospectively the low level of corrected cells can be explained by the lack of BM conditioning. Gene-transduced CGD cells are not expected to have a proliferative advantage over non transduced cells. Engraftment of a sufficient high number of HSCs to provide long-term correction is probably only possible when BM conditioning is performed or when a resistance gene is co-expressed to allow for *in vivo* selection. In animal studies sufficient gene marking associated with expression at therapeutically relevant levels in both lymphoid and myeloid lineages after GT could only be reached if the protocols contained non-myeloablative conditioning[62, 63], myeloablative conditioning[52, 53, 55, 57], sub-myeloablative or chemotherapy doses [64, 65].

The first human clinical GT trial for X-CGD with BM conditioning was performed by the group of M. Grez, in Frankfurt, Germany, reviewed by[66]. In this study, one X-linked CGD patient got a mild myelosuppressive conditioning regimen (cyclophosphamide $1 g/m^2$/day on four consecutive days) prior to reinfusion of transduced cells. A bicistronic vector containing

gp91^{phox} cDNA and a marker gene encoding a cytoplasmic truncated form of the human low-affinity nerve growth factor receptor (ΔLNGFR) was used. Despite a high transduction efficiency (44%) in G-CSF-mobilized peripheral blood CD34+ cells the engraftment of gene-transduced cells was still minimal (1% of the peripheral blood granulocytes after reinfusion, stable for 3 months). Possibly the immunosuppressive drug cyclophosphamide was not sufficient to generate niches in BM and to allow engraftment of cells.

Then, GTs for the correction of severe combined immunodeficiency (SCID)-X1 and adenosine deaminase (ADA)-SCID successfully addressed the topic of engraftment[67, 68]. These studies showed proof-of-principle for the correction of immunodeficiencies by GT. At the same time the SCID-X1 study revealed limitations and risks associated with this new technology. The outcome of the ADA-SCID GT was particular relevant for future CGD studies, since BM conditioning was used for the first time to improve engraftment and survival of gene-transduced cells[67]. The two patients enrolled in this study[67] received busulfan (2mg/kg/day) for 2 days before infusion of transduced cells. Busulfan is a chemotherapeutic drug, which is well tolerated and widely used in pediatric patients receiving HSCT[69]. The busulfan treatment resulted in high and long-time engraftment (Patient 1: 10% gene-marked cells in CD34+ progenitor cells 1 year after injection), in an increase in lymphocyte counts and in improved immune functions and clinical status.

Most relevant with regard to CGD GT studies was the achievement of substantial amounts of gene-marked myeloid cells, e. g. granulocytes.

The outcomes of the two SCID GT studies paved the way for a new GT study for X-CGD which was performed in Frankfurt/Zurich in two patients in 2005[4]. Two modifications in the GT protocol were included: First, improved gene transduction protocol by using high concentrations of Flt3 ligand and stemcell factor in combination with thrombopoietin and IL-6 on retronectin-precoated culture bags. Second, BM conditioning by busulfan for better engraftment of gene-transduced cells. A monocistronic γ-retroviral vector encoding gp91^{phox} (SFgp91phox) was used to transduce CD34+ cells which were isolated by G-CSF mobilization from peripheral blood of patients. Before reinfusion of transduced cells, myeloablative conditioning of BM was performed by busulfan (4mg/kg/day). The outcome of this GT was successful: For the first time high levels of gene marked neutrophils (range from 12% to 31% of gp91^{phox} positive blood granulocytes 4 to 5 months after GT) could be detected. Up to day +120 comparable amounts of functionally corrected neutrophils were measured in functional assays in both patients. Refractory bacterial and fungal infections from which patients had suffered for many years were cleared after GT. Surprisingly, an increase in the number of gene-corrected cells of up to 50 to 60% of all peripheral blood granulocytes was observed in both patients starting 5 months after transplantation and remained at this level. The overall myeloid cell proliferation was not affected by this increase,

so the granulocyte numbers remained constant as before GT. Retroviral integration sites (RIS) in the expanded cell population were analyzed by linker adapter-mediated polymerase chain reactions (LAM-PCR). Integration hot spots were found in three growth-promoting genes, namely *mds1-evi1*, *prdm16*, *setbp1*. The activation of these three genes raises concerns about possible occurrence of uncontrolled proliferation, abnormal hematopoiesis and eventually leukemogenesis in such cell clones. One of these hot spots, *evi1* (ecotropic viral integration 1 site) encodes a transcription factor (TF) with a role in both self-renewal and transformation of HSCs. Constitutive overexpression of *evi1* in murine BM cells induces a fatal pancytopenia with hypercellular BM and peripheral cytopenia. These features resemble those reported in the human *evi1*-positive myelodysplastic syndrome (MDS). However, none of the animals progressed to acute leukemia and the death of the animals was attributed to pancytopenia[70]. Evi1 overexpression itself is insufficient to induce acute myeloid leukemia (AML). The progression from MDS to AML requires additional genetic events. Watanabe-Okochi and coworkers[71] recently identified a mutation in *aml1* which collaborates with *evi1* overexpression to induce MDS/AML in mice. In human, the *evi1* overexpression is associated with poor survival of AML[72, 73]. However, mice experiments have revealed that single *mds1-evi1* integrations resulted in long-term *in vivo* clonal dominance without turning leukemic[74]. Moreover, in a nonhuman primate gene-marking study, the *mds1-evi1* gene was found to be a common integration site (CIS) with long-term clonal activity without clonal expansion and signs of leukemia[75]. Moreover, immortalized myeloid progenitor cell lines with RIS in *evi1*, *prdm16*, *setbp1* did not engraft or result in leukemia in irradiated mice[76]. BM cells isolated from the patient 1 of this X-CGD gene therapy study did not show malignant transformations. The BM cells of the patients were strictly dependent on cytokines for growth and did not engraft in xenograft animal models[4]. Nevertheless, the proliferative stress caused by overexpression of *mds1/evi1* or *prdm16* in hematopoietic progenitor cells may cause further mutations that might develop into a malignant hematopoiesis. A long-term follow-up of treated X-CGD patients must be done to draw conclusions on efficacy and safety of this procedure.

Risks of retroviral GT: Insertional mutagenesis and its consequences

For a long time the integrations of retrovirus and retrovirus-based vectors was believed to be distributed randomly throughout the genome. A malignant outcome of single insertions in the context of clinical settings was considered to be extremely unlikely[77]. However, first published reports of serious adverse events (SAEs) in retroviral GT studies changed this view completely[78-80]. SAEs were for the first time observed in an otherwise successful clinical GT trial in the X-SCID study. Three years after successful correction of SCID-X1 two patients suffered from uncontrolled lymphoproliferation or in clinical terms from lymphoblastic leukemia. The uncontrolled lymphoproliferation resulted from clonal T-cell expansion as a consequence of retroviral insertional activation of the *lmo2* gene, which was previously implicated in *de novo* acute lymphoblastic leukemia[81]. Gene transfer vectors had based on the MLV which were used clinically to transduce HSC since the early 1990s[82].

Baum et al. discussed in several interesting reviews the chances and risks of retroviral GT, especially in a hematopoietic setting[79, 80, 83, 84]. For the first time they discussed side-effects which could occur by performing genetic manipulations in HSCs. Three side-effects are summarized in the following:

1. Selective loss of long-term HSC properties: For genetic modification by viral transduction, HSCs have to be cultured and enriched *ex vivo*. The physiological proliferation of HSCs in BM is known to be slow. Since the efficiency of γ-retroviral gene-transfer is cell cycle dependent, HSCs need to be stimulated by a cocktail of cytokines to induce proliferation. This *ex vivo* culture harbors the danger of a selective loss of long-term HSC properties. Present attempts to optimize the culture conditions focus on the cytokine cocktail and the reduction of the culture period.

2. Genotoxicity: Genotoxicity rising from transgene insertions, called insertional mutagenesis, are one feature of all stable gene transfer methods. Retroviral insertion has been studied in great detail (see below). The incidence of SAEs after retroviral insertions have been previously estimated to be rather low (between 10^{-6} and 10^{-8} per insertion event). However, based on the hypothesis of a semirandom choice of target sequence for retroviral integration, proto-oncogenic activation by RIS would be expected to be more frequent (10^{-2} and 10^{-3} per insertion event within 10 kb of a potential proto-oncogene)[79]. But several biological filters lower the risk of such insertion events from being directly cancerogenic. RIS are mostly monoallelic which reduces the risk for mostly recessive mutational events. RIS could lead to signal alterations which reduce the probability of survival of affected cell clone by triggering differentiation, apoptosis or impeding engraftment. A single mutation is normally not sufficient to develop a malignant phenotype. To promote uncontrolled proliferation of transformed

clones, several cooperating genetic mutations and environmental stimuli are required as observed in clinical and experimental findings of cancer development. For example, induction of leukemia is often caused by cooperation of at least two genetic alterations: one disturbing differentiation, another promoting proliferation. Thus, there must be a distinction between the frequency of insertional hits in (vicinity of) cellular growth regulatory genes, the frequency of functional gene alterations after insertional mutagenesis, and the frequency of insertional oncogenesis. In general, SAEs have been observed under conditions of high multiplicity of infection. From that point of view, SAEs by retroviral integrations can be markedly reduced by transfer of not more than 1 (or 2) transgenes per cell. The specific type of retroviral vector (backbone) utilized for gene-transfer influences the risk to activate neighboring cellular sequences. The long terminal repeat (LTR) of conventional retroviral vectors with its strong promoter and most relevant enhancer activities and the relatively weak polyadenylation signal can activate cellular sequences located downstream of the transgene insertion site. Therefore vector types with deleted enhancer elements after integration are likely to reduce the risk of activation of adjacent genes after integration.

3. Immune response: SAEs can also occur by innate or acquired immunity against vector components or gene-modified cells. Especially in nonmyeloablative conditioning the tolerance could be incomplete. For example a GT approach to correct hemophilia B in mice by using a lentiviral vector encoding coagulation factor IX resulted in priming of an anti-F.IX immune response and rapid clearance of gene-modified cells[85].

HSC and hematopoiesis

Definition of HSCs

HSCs are by definition clonogenic cells which possess the ability to self-renew and the ability to differentiate into mature blood cells of all lineages. By these abilities they contain the capacity to regenerate the whole blood system for a lifetime of an organism.

Murine HSC and hematopoiesis

The Weissmann group first characterized HSCs in the murine system as cells that do not express any lineage specific antigens (Lin^- cells), that are positive for the stem cell associated antigen (ScaI), and express low amounts of T-cell antigen Thy-1[86]. In later experiments, mouse HSCs were characterized as Lin^-ScaI+c-kit+ (LSK) cells, which can be further distinguished in clonogenic longterm self-renewing HSCs (LT-HSCs), transiently self-renewing HSCs (short-term HSCs) and non-self-renewing multipotent progenitors (MPPs)[87]. Downstream of the LSK compartment, cells loose their multipotent features and become lineage progenitors. The developmental abilities of these progenitors are either restricted to the myeloid lineage, designated as common myeloid progenitors (CMPs) or to the lymphoid lineage, designated as common lymphoid progenitors (CLPs), respectively. CLPs can further differentiate to T-lymphocytes, B-lymphocytes and natural killer (NK) cells, whereas CMPs can progress to granulocytic cells (neutrophils, eosinophils, basophils), monocytes, macrophages, erythrocytes and megakaryocytes[88] (figure 4). From the discovery of CMPs and CLPs it was concluded that at this developmental stage all hematopoietic cells become either restricted to the myeloid or lymphoid development[88]. However, cells with lymphoid developmental capacities have been found to possess the capacity to give rise to granulocytes or macrophages (reviewed by[89]). Cells with these abilities do not fit into the classical hematopoietic model (figure 4). From these observation it was concluded that there are alternative developmental pathways. An alternative scenario would be that cells which were once committed to a certain lineage are not necessarily determined and might still retain some developmental flexibility.

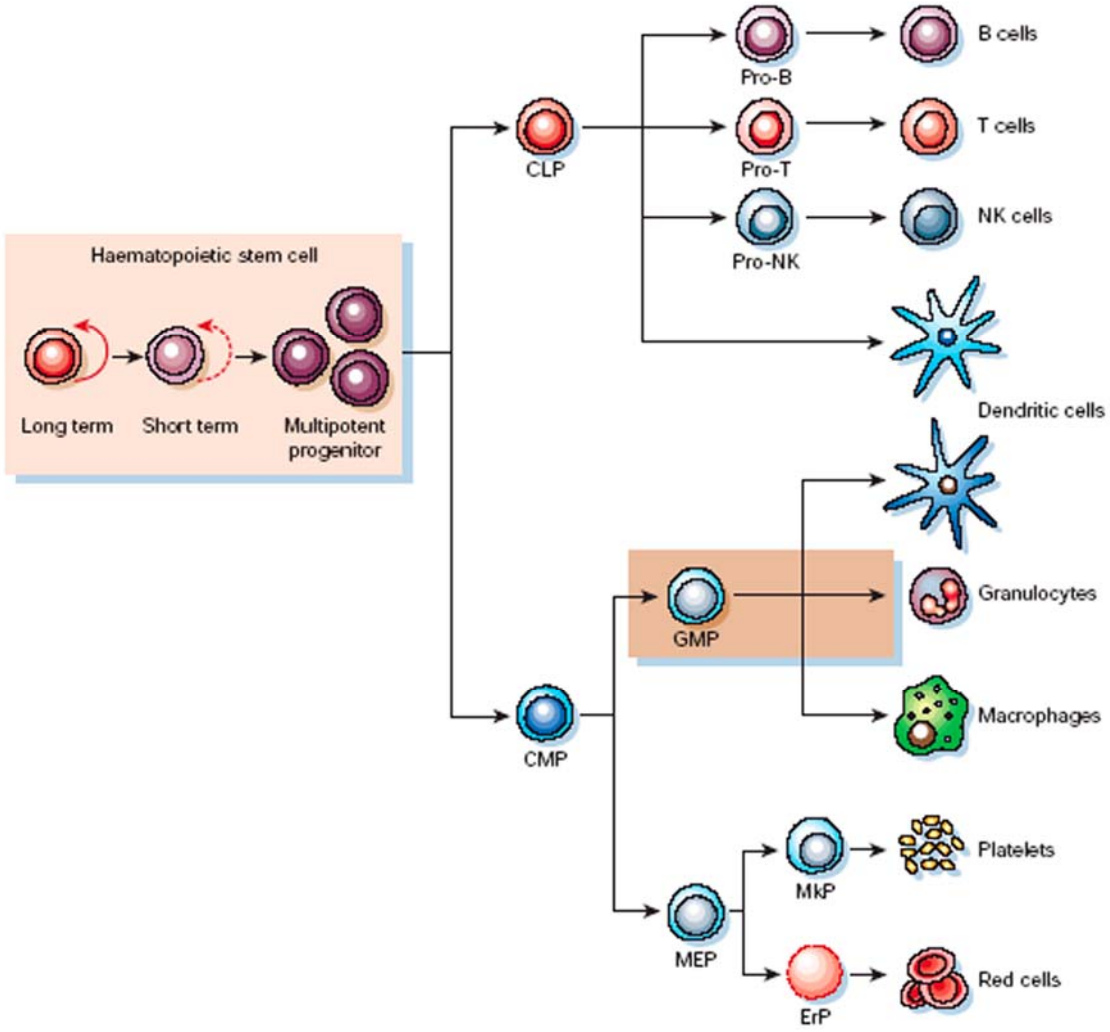

Figure 4 Classical model of hematopoiesis: All blood cells originate from a common longterm self-renewing HSC. HSCs can be subdivided into LT-HSCs, short-term HSCs and non-self-renewing MPPs. They give rise to CLPs and CMPs. CLPs mature to B-lymphocytes, T-lymphocytes and NK cells, whereas CMPs propagate to granulocytic cells (neutrophils, eosinophils, basophils), monocytes, macrophages, erythrocytes and megakaryocytes. ErP, erythrocyte precursor; MEP, megakaryocyte erythrocyte precursor (modified from [88]).

Hematopoietic lineage specification

Although HSCs have been investigated most intensively, the nature of factors which drive HSCs either to self-renewal or differentiation remains largely unknown. Both uncontrolled expansion as well as loss of stem cells would be fatal for multicellular organisms. Two observations indicate that the development of HSCs is orchestrated by a combination of extrinsic and intrinsic signals. First, HSCs have been found to be regulated by specialized microenvironments, the hematopoietic niches. In the endosteum of the BM osteoblasts are regulating key elements (influence stem cell function through notch signaling pathway) and in vascular niches (found in the spleen and BM) sinusoidal endothelial cells play a pivotal role[90]. Second, HSCs and hematopoetic progenitor cells were reported to have the ability to divide asymmetrically[91]. During asymmetric cell division cells are polarized and localize specific

molecules to distinct regions of the cell, which are then transmitted unequally to the daughter-cells.

The combination of these extrinsic and intrinsic signals is believed to alter the genetic program of the corresponding hematopoietic cells. Baum et al[92] simplified the developmental hierarchy of hematopoiesis to three levels: a first level containing the long-lived multipotential stem cells with multilineage expression at low level, reduced metabolic activity, mainly in the G_0 phase of the cell cycle, ready for activation of proliferation and differentiation; a second level of continuous differentiating progenitor and precursor cells with high metabolic activity, fast cell cycle, high *de novo* synthesis of RNA, increasing number of genetic loci irreversibly inactivated upon differentiation; a third level of mature cells in blood, lymphoid organs and tissues with short half-live (e.g. granulocytes, red cells and platelets) or upon activation re-entering cell cycle and persisting for long-time (e.g. macrophages, mast cells, lymphocytes), with no *de novo* RNA synthesis or restricted to lineage-specific genetic programs.

Human HSC

BM was transferred already in the early 1960s to restore the blood system in immunocompromised patients. However, it is difficult to characterize defined cell populations within BM containing true HSC activity in humans. For ethical reasons human cell fractions cannot be tested experimentally whether or not they contain HSCs or more mature progenitor cells that are able to mediate longterm engraftment (reviewed by[89]). Nowadays, knowledge about human HSCs is mainly gained by xenografts experiments in NOD-SCID mice. The most primitive human hematopoietic cells are characterized by Lineage negative (Lin-) CD133+ CD34- CD38- CD33-[93] further progressing to Lin- CD34+ CD38- CD90+ CD45RA-expression[94, 95]. A down regulation of CD90 was reported as marker for a reduced self-renewal potential. The cells are able to restore longterm hematopoiesis, but show a significant reduction in engraftment. The next developmental step is then the upregulation of CD45RA. These cells are unable to restore human hematopoiesis in mouse.

Native retroviruses

General description

The family of retroviridae are divided in three subfamilies: oncoviridae, lentiviridae and spumaviridae. The retroviral life cycle is characterized by transcription to single stranded RNA (ssRNA), packaging of two ssRNA molecules into one infectious particle, the reverse transcription into DNA and integration into the target genome. Retroviral particles contain three proteins with enzymatic activity: the reverse transcriptase (RT); the viral integrase (IN), (that catalyzes the insertion of the viral DNA into host cell DNA) and the viral protease (PR) which cleaves at specific site to produce mature virion proteins. Most retroviruses from oncoviridae (alpha-, beta-, and gammaretrovirus) contain defined *simple* genomes encoding only three genes common to all retroviruses (figure 7): *pol*, which encodes the enzymes (RT, associated RNase H and IN), *gag* (group-specific antigen gene) which encodes proteins of the viral core (matrix and capsid proteins) and *env* which encodes the viral transmembrane envelope. Envelope proteins determine the host cell specificity. Their classification into ecotropic, xenotropic and amphotropic strain originated from early reports on virus replication in cell culture[96]. Mice-tropic or ecotropic strains replicated only in culture of mouse and rat cells. MLV strains that replicated not in mouse cells but in cultures derived from other species were called xenotropic strains. The third group, the amphotropic MLVs have general host range characteristics of both mouse and xenotropic viruses.

Retroviral life cycle

The retroviral life cycle follows the general pathway of enveloped viral infections (figure 6). However, since the viral genome consists of ssRNA, the genetic information must be transferred into double-stranded DNA (dsDNA) before integration can take place (see textbooks for more details, e.g.[97, 98]).

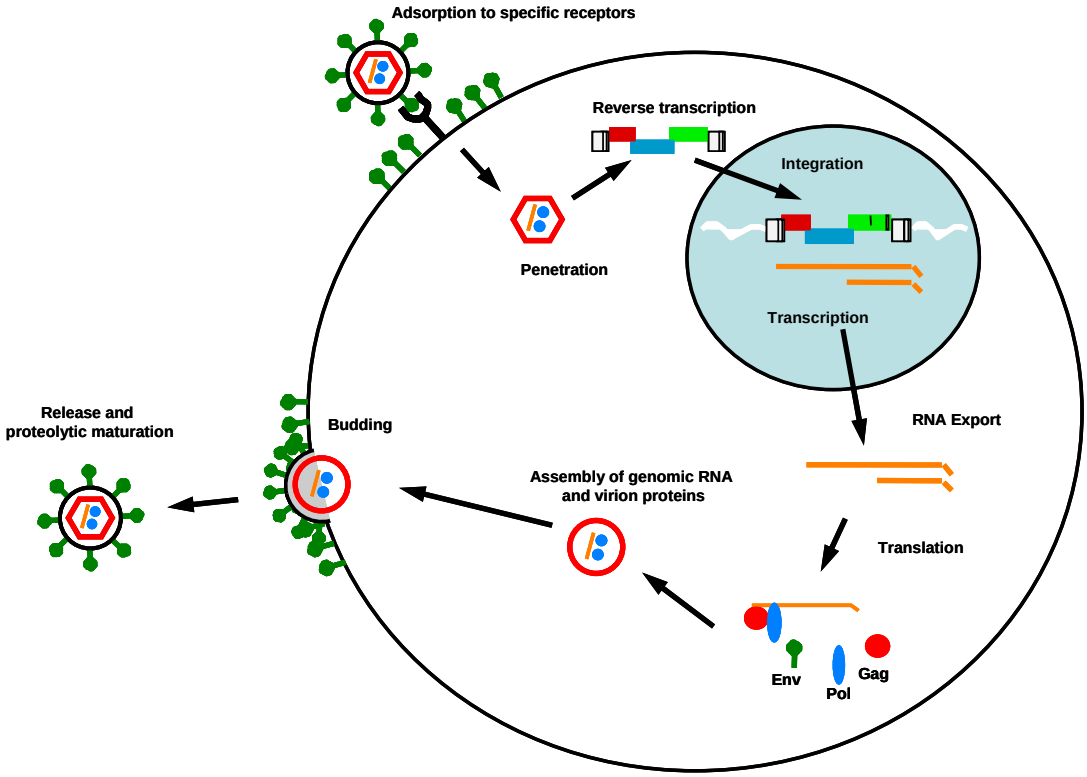

Figure 6 Retroviral life cycle. The cycle is initiated by adsorption of the viral envelope to a specific receptor at the surface of the host cell. After fusion with the plasma membrane, the viral capsid or core enters the cell and the RNA genome is reverse transcribed into viral dsDNA which passes the nucleus and integrates into the host genome. The viral DNA serves as template for RNA molecules encoding the necessary viral proteins and progeny RNA packaged into new particles. The assembly and release by budding occurs at the cell membrane and fully infectious particles are generated by proteolytic maturation (modified from[97]).

In this study retroviral vectors derived from MLV were used for gene transfer. Therefore the retroviral MLV genome is introduced in the following section.

The coding sequences are flanked by LTR. LTR contain several transcriptional control elements: promoter, enhancer, polyadenylation signals and sequences involved in replication and integration (figure 7). The U3 region includes the promoter and multiple enhancer sequences which interact with cellular transcriptional activator proteins. Transcription initiates at the boundary between U3 and R and the site of poly-adenylation is found at the boundary between R and U5. The (+)-DNA-strand initiation site is called polypurine-tract (PPT), whereas (-)-DNA-strand initiation site is called primer binding site (PBS). ψ signal region is responsible for packaging of viral RNA into capsid. The Gag and Pol proteins are first translated as polyprotein precursors from the full length transcript and afterwards cleaved to mature proteins by PR. In contrast, the envelope polypeptide is translated from spliced genomic transcript and further processed by cellular host enzyme machinery.

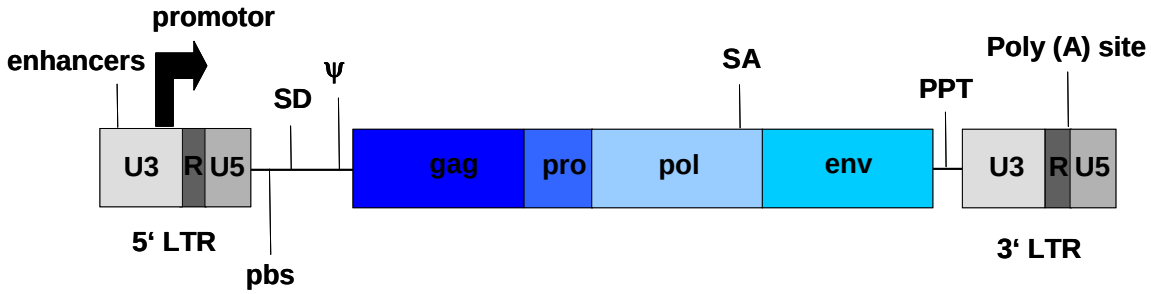

Figure 7 Proviral genome of retroviruses (e.g. MLV). The LTR includes promoter and enhancer regions (U sequences unique to the 5' (U5) or 3' (U3), repeat region (R)). Polyadenylation site (poly A) site lies within the 3 'LTR between R and U5 region. The shown *cis*-acting elements are tRNA primer binding site (pbs), the splice donor site (SD), the packaging signal (ψ) and the PPT. The shown *trans*-acting elements are the coding regions group-antigen specific gene (*gag*), protease gene (*pro*), polymerase gene (*pol*) and envelope gene (*env*).

Other retroviruses, like the HIV-1 lentivirus, have more complex genomes which include additionally auxiliary or accessory genes encoding non structural proteins[98]. These accessory proteins affect viral gene expression and/or pathogenesis.

Replication and integration of retroviral genome

After the capsid is released inside the host cell, viral RNA is reversely transcribed in double stranded DNA-RNA chimera and further processed to dsDNA. This reverse transcription is a complex process in which the (-)-strand DNA and afterwards the (+)-strand DNA is synthesized by RT[98]. The produced viral dsDNA forms a complex with viral-encoded integrase and other proteins (preintegration complex (PIC)). This PIC is transported from the cytoplasm into the nucleus. Mechanisms mediating nuclear import of retroviral genomes are poorly understood[99]. It is known that γ-retroviruses like MLV and lentiviruses like HIV-1 enter the nucleus by different mechanisms[100, 101]. Successful MLV proviral DNA integration into the target genome was only observed in dividing cells, whereas HIV-1 infection was observed in dividing and also non dividing cells. Possibly the large intracellular complexes of MLV can not be transported actively to the nucleus, so they gain only access to the host chromosome after breakdown of the nuclear membrane in mitosis. However, up to now it can not be excluded that in MLV nuclear transport other mechanisms might be involved as well[99]. After nuclear import, viral DNA is inserted into the host cell genome, a process termed integration. Historically, integration events of retroviruses were believed to be randomly distributed throughout the genome. However, gene therapy studies with MLV derived vectors showed malignant transformations after two independent insertions near the same growth-promoting gene *lmo2*[78]. For MLV and MLV-based vectors a preference of integration events have been observed in the vicinity of transcription start regions [102], of CpG islands and of DNaseI-hypersensitive sites. Different integration patterns were found for HIV-1 with HIV-1 preferably integrating into the coding sequences[103].

Retroviruses as gene delivery systems

An ideal GT vector to cure a genetic defect within hematopoiesis should integrate into the host genome of longterm repopulating cells. The integration should result in transgene expression for the entire life span of the cell and its progeny without causing severe side effects. For gene transfer into HSC retroviral vectors are attractive tools. Historically, retroviral vectors were the first viral system tested in a gene marking study in 1989 and applied in the first clinical GT trial in 1990[104]. They lead to stable longterm integration into the HSC genome. They are "safe" vector systems since viral genes responsible for spreading of viral infections can be eliminated. They integrate at predictable copy number per cell. They can transfer about 7 to 9 kb of foreign DNA which is sufficient for most therapeutic genes.

The most frequently used vector system for HSC transduction in clinical trials is derived from the MLV genome. The retroviral vectors used in GT applications are replication defective. In these vectors the genetic information elementary for virus production in infected cells was deleted. Instead, the coding gene regions (*gag*, *pol*, *env*) which are necessary for generation of retroviral particles are provided *in trans* during virus production. Packaging cell lines have been produced by stably integration of plasmids encoding the *gag/pol* and/or *env* open reading frames (ORF) to provide corresponding gene products *in trans* during virus production. Nowadays, there are several different packaging cell line systems available (reviewed by[105]). By changing virus envelope proteins (encoded by the *env* gene) the target cell specificity of the viral vector can be modified. For clinical HSC transduction, vectors pseudotyped with amphotropic MLV- or gibbon ape leukemia virus (GALV) envelope have been used.

In the last 15 years much progress has been achieved in the development of viral vectors derived from lentivirus (HIV-1). In comparison to γ-retroviral vectors, lentiviruses have the advantage that they are able to integrate into a variety of nondividing cells[100]. This would be particular attractive for HSC transduction since true stemcells are predominantly dormant (reviewed by[106]). However, the transduction of non-proliferating CD34+ cells from X-CGD patients by HIV-1 derived lentiviral vector resulted only in low transduction rates, whereas highest transduction rates could be observed after stimulation of cell proliferation by multiple cytokines[107]. Another very important issue for lentiviral vectors is the biosafety with respect to the pathogenicity of the parental virus. The wild type HIV virus encodes the *vif*, *vpr*, *vpu* and the *nef* ORFs. Their gene products are dispensable for virus production but are involved in HIV pathogenesis. In third generation lentiviral vectors these ORFs were deleted, which added to the safety of these vectors[108-110]. The development of so called self-inactivating (SIN) vectors has further improved the safety of MLV and HIV-1 based vectors[111]. The strong enhancers in the viral LTR of non-SIN retroviral vectors have a high potential to transactivate genes adjacent to the integration site. SIN vectors contain a deletion of 299 base pairs in the

3’ LTR including the promoter and enhancer sequences. Upon reverse transcription into dsDNA this deletion is copied to the 5’ LTR which results in transcriptional inactive proviral LTRs in the host cell genome. Desired transgene expression has to be driven by an additional internal promoter. Cell culture assays[76] and *in vivo* assays[112] revealed that a SIN vector containing strong internal enhancer/promoter showed significantly lower frequency of transformation by insertional mutagenesis than their LTR-driven counterparts[113]. A further reduction in transactivation activity was achieved by replacing the strong viral internal promoter/enhancer against a weaker cellular promoter[114]. In SIN vectors, the use of a tissue-specific promoter allows to restrict the transgene expression to a certain cell population and limits transactivation to these cells. The risk of a single transactivation event to add to malignant transformation is more pronounced in stem- and progenitorcells than in terminally differentiated cells. In the French X-SCID trial *lmo2* transactivation contributed to the transformation of T lymphocyte precursors[78]. Hence, the risk of leukomogenesis can be reduced significantly by restricting the promoter activity to terminally differentiated cells. Indeed, recently two internal cellular promoters (elongation factor-1 alpha (EF1α) and phoshoglycerate kinase) cloned in SIN vectors greatly decreased the risk of insertional transformation in cell culture systems. Even using multiple copies of the of EF1 α SIN per cell did not activate the crucial cellular proto-oncogene *evi1*[114]. On the other hand, the use of a weak cellular promoter can result in low transgen-expression[115]. Further improvements can be achieved at the post-transcriptional level. The nuclear export of RNA is known to be a rate limiting step[116]. For example, the introduction of an additional element, the so called woodchuck hepatitis virus post transcriptional regulatory element (WPRE) enhanced retroviral vector titer and expression[117]. WPRE facilitates nucleocytoplasmic transport of RNA[118]. Taken together, these studies underline the great impact of vector design on genotoxicity.

Foot-and-mouth disease virus 2A co-expression technology

Picornaviruses, such as poliovirus and foot-and-mouth disease virus (FMDV) encode all their proteins within one single ORF. Individual virus proteins are derived from the polyprotein precursor by (auto)proteolytic processing. In the case of the FMDV and some other picornaviruses, the oligopeptide 2A (approx. 20 amino acid) plays a major role in polyprotein processing (reviewed by[119]). It represents a autonomous element capable of mediating „cleavage“ at its own C terminus together with the N-terminal residue of protein 2B (a conserved proline residue)[56]. The 2A region is not a proteinase; rather it is thought to mediate a ribosome skipping, a novel type of cotranslational, intraribosomal cleavage[120]. The majority of 2A and 2A-like peptides seem to form an amphipathic helix whereas the amino acids next to the „cleavage“ site form a tight turn[121]. This conformation of 2A places strain on the peptidyltransferase center of the ribosome and prohibits a nucleophilic attack by the incoming (prolyl)-tRNA amide nitrogen that normally creates the new peptide bond. A part of the ribosome then cease the translation process, while the other part of the ribosome continues the translation process and thereby effectively initiates a second polypeptide chain by the prolyl-tRNA to produce the downstream part as a discrete protein[121, 122].

The 2A methodology is an attractive tool for *in vivo* co-expression, because it only needs eukaryotic ribosomes to be active[56]. Multiple proteins can be co-expressed from a single mRNA by fusing multiple ORFs with intervening 2A sequences into a single, long ORF. The resulting single polyprotein self-processes into multiple products.

In several studies, proteins with a signal sequence for post-translational targeting have been linked by the 2A polypeptide. In these constructs 2A mediated cleavage did not interfere with the natural localization to the nucleus, chloroplast, mitochondria, membranes or cytosolic tubules (reviewed by[119]).

Felipe et al[120] presented a model of co-translational cleavage in which one protein chain is directed into the endoplasmic reticulum (ER) and the other protein is synthesized in the cytosol (figure 8). The N-terminal portion of the cleaved polyprotein contains an ER signal sequence. The nascent peptide chain passes across the ER membrane. The synthesis of the N-terminal protein into the lumen of the ER is terminated by the 2A cleavage activity. The remaining C-terminal part of the polyprotein is translated irrespective of the signal sequence of the N-terminal part. Depending on whether or not an additional signal sequence is present, the translocon either re-opens to allow passage into ER or remains closed, resulting in the cytosolic localization or in the synthesis into the ER of the second C-terminal part of the original polyprotein.

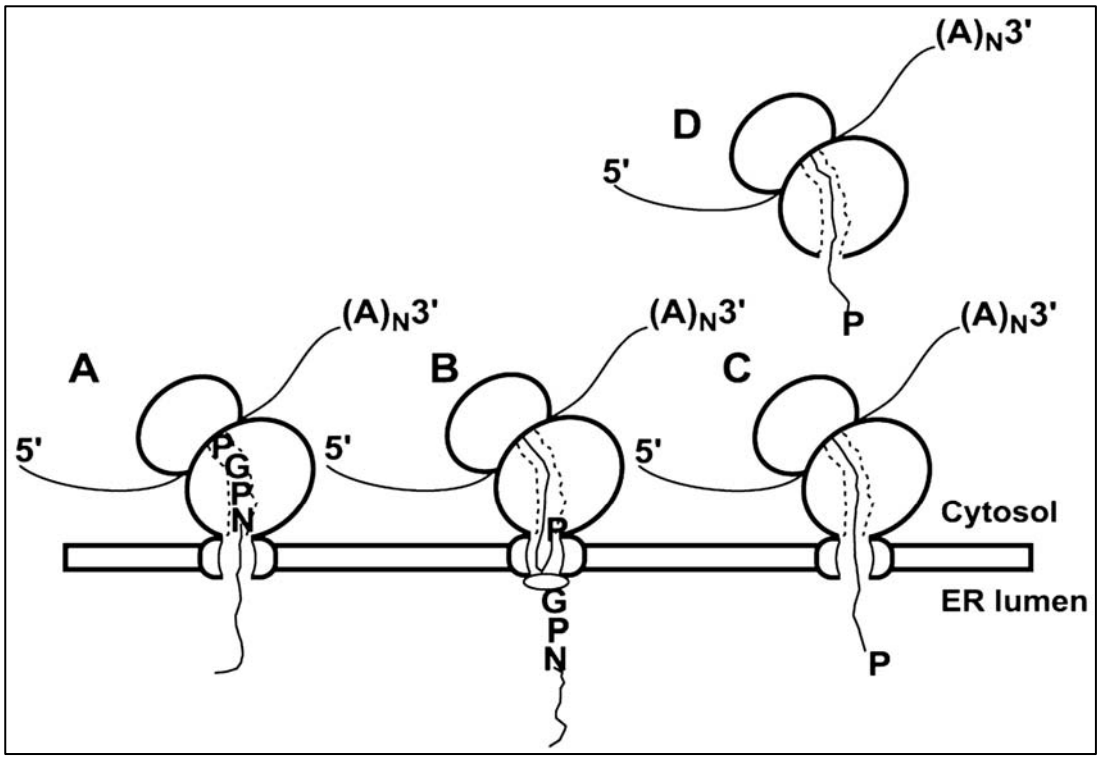

Figure 8 Co-translational translocation and intraribosomal nascent chain cleavage by 2A (see text for details; taken from[120]).

An alternative approach would be the use of internal ribosome entry sequences (IRESes). IRES sequences are able to direct ribosomes to initiate the translation of a second ORF at internal sites within the mRNA. Unfortunately, the Cap-initiated translation and IRES-initiated translation show different efficiencies. IRES-mediated translation is reported to result in a much lower expression level than the Cap-initiated counterpart. At limiting multiplicity of infection (MOI), expression in a bicistronic vector based on 2A was approx. 4 times greater than that of an IRES based vector[123]. Furthermore, problems can arise from competition between different IRESes[124]. Compared to IRES, the 2A methodology offers several advantages. In viral vectors the packaging capacity is limiting[125]. The 2A sequence (66nt) is much shorter than the IRES (~500nt). Most important, the mechanism of the 2A-mediated co-expression dictates the synthesis of two encoded proteins in a molar ratio of 1:1.

2A functions in all eukaryontic systems, but not in prokaryotic systems[122], and has already been applied to a broad range of biotechnical applications (reviewed by[119]). GT vectors incorporating 2A have been designed to deliver suicide genes[126], to express transgenes in oncolytic adenoviruses[127] and to quantify the expression levels of a nuclear homeoboxgene HoxB4 in hematopoietic stem- and precursor cells by coexpressing a cytoplasmic reporter gene, such as the enhanced green fluorescent protein (GFP)[128].

Scope of the thesis

CGD comprises a group of primary immunodeficiencies characterized by the failure of respiratory burst and thereby an impaired antimicrobial activity of phagocytes. 60% of all CGD patients suffer from mutations in the X-linked *cybb* gene, followed by 30% with a mutation in the *ncf1* gene encoding p47^{phox} [1, 13]. First clinical success could be achieved in a recent (γ)-retroviral X-CGD gene therapy (GT) study[4]. This therapy represents a therapy complementary to BMT, which is associated with unacceptable high rates of morbidity, mortality and graft failure, except in very selected cases if a HLA-identical donor is available[129, 130]. However, retroviral GTs for X-CGD and ADA-SCID[78] were accompanied with clonal dominance or leucemic progression as long-term effects of transactivation events.

One option to minimize the risks for transactivation in CGD GT is the use of γ-retroviral SIN vectors[111, 131], in which the viral promoter/enhancer elements are inactivated upon integration. The transgene expression is driven by an internal tissue-specific promoter element which ideally lacks enhancer activity and restricts transgene expression to terminally differentiated granulocytes/monocytes.

For the p47^{phox}-deficient form of CGD, BMT is the only therapy available at present. Therefore, the intended project aims to develop of a safer γ-retroviral SIN vector for gene replacement therapy to correct the p47^{phox} CGD phenotype.

Three major aims were formulated:

First: To develop a SIN vector for p47^{phox} CGD gene therapy we conducted two rounds of screening to find a potent myelospecific promoter which induces p47^{phox} transgene expression upon granulocytic differentiation (chapter 1).

Second: The best promoter candidate was tested in an *in vivo* GT in p47^{phox} -/- mice to confirm its myelospecificity *in vivo*. In this *in vivo* experiment the myelospecificity of the p47^{phox} transgene expression was compared to a GT conducted with a control vector containing the strong constitutive SFFV promoter (chapter 2).

Third: The detection of p47^{phox} expression is a mandatory requirement for GT monitoring. Intracellular immunostaining of the cytoplasmic p47^{phox} transgene product interferes, however, with the analysis of respiratory burst activity. To overcome this limitation we developed a new reporter system for measuring cytoplasmic p47^{phox} by co-expression of a cellular surface marker in a molar ratio of 1:1 (chapter 3).

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Chapter 1:

New myelospecific self-inactivating gammaretroviral vectors for gene therapy of $p47^{phox}$ - deficient form of chronic granulomatous disease

written as manuscript for publication

Contributions: All experiments were conducted by Vital Wohlgensinger in Zürich. Vital Wohlgensinger and Ulrich Siler analyzed the results. All figures in this chapter were designed by Vital Wohlgensinger. Ulrich Siler designed the research.

Abstract

First clinical success in retroviral gene therapy (GT) could be achieved for X-linked chronic granulomatous disease (X-CGD), severe combined immunodificiency (X-SCID) and adenosine deaminase severe combined immunodificiency (ADA-SCID), but were accompanied with clonal dominance or leukemic progression as long term effects of transactivation events. To develop a GT vector for the $p47^{phox}$-deficient form of chronic granulomatous disease (CGD) we therefore cloned various myelospecific promoter candidates into gamma (γ)-retroviral self-inactivating (SIN) vectors driving $p47^{phox}$ expression. The constructs were screened for myelospecific expression and functional reconstitution of the NADPH oxidase activity in transduced murine $p47^{phox}$ -/- hematopoietic stem cells (HSC) upon propagation to granulocytes in cell culture. By this screen we have identified a new, potent myelospecific promoter, microRNA-223 (miRNA-223), which results in $p47^{phox}$ expression exclusively in granulocytes. Furthermore, miRNA-223 controlled $p47^{phox}$ expression resulted in functional reconstitution of the NADPH-oxidase in cell culture.

Introduction

CGD results from an impaired respiratory burst activity caused by an inactive phagocyte NADPH oxidase complex[1]. This enzyme complex consists of two membrane spanning subunits ($gp91^{phox}$ and $p22^{phox}$) plus three cytosolic components ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$). Approximately 60% of all CGD cases were estimated to result from mutations in the X-linked *cybb*-gene (X-CGD) encoding $gp91^{phox}$ followed by additional 30% caused by mutations in the *ncf1*-gene encoding $p47^{phox}$ [2]. Unfortunately, hematopoietic stem cell transplantation (HSCT) for CGD has been associated with unacceptably high rates of morbidity, mortality and graft failure, except in very selected cases when a human leukocyte antigen (HLA) -identical donor is available[3, 4]. A complementary therapeutic approach for CGD patients is the genetic correction of autologous HSCs. CGD could be successfully corrected in animal models by GT[5, 6]. Furthermore, a recent GT trial aimed to correct X-CGD has temporarily proved clinically benefit to the patients[7]. However, oncogene transactivation by viral long terminal repeat (LTR) sequences resulted in clonal dominance or leukemic progression[7 8]. These adverse events highlighted the risks associated with integrating viruses as vectors for gene delivery[9]. To minimize the risks for transactivation, we have developed a series of SIN γ-retroviral vectors for $p47^{phox}$ GT. SIN vectors contain a deletion within the U3 region of the viral 3' LTR which results in the inactivation of the viral promoter/enhancer elements upon reverse transcription[10]. Transgene expression is driven by an internal tissue-specific promoter element which ideally lacks enhancer activity. E. g. a myelospecific promoter restricts the transgene-expression to terminally differentiated granulocytes/monocytes and

thereby prevents transactivation events in HSC. In the literature several promoter sequences have been described which resulted in myelospecific gene expression. Table 1 summarizes some myelospecific promoter elements which were tested in our study.

Myeloid promoter	Transcription factor (TF) binding sites	Description	Size, bp
human $p47^{phox}$ promoter	PU.1[11],ISRE[12], HBP1[13], Sp1[14], PEA3[11], PEBP2[11], Ets-1[11]	Regulates myelospecific human $p47^{phox}$ expression: $p47^{phox}$ expression is mainly found in myeloid cells, e.g. neutrophils, monocytes, but also in B-cells. Only 58bp of the proximal $p47^{phox}$ sequence including PU.1 TF binding site were reported to be sufficient to direct significant reporter gene activity in myeloid cells[15].	3050[11]
human $p40^{phox}$ promoter	PU.1[16]	Restricts human $p40^{phox}$ expression to myeloid lineage; proximal 106 base pairs of 5'-flanking region of the human $p40^{phox}$ gene exhibited maximum myeloid promoter activity[16].	106[16]
c-fes promoter	SP1, PU.1 and unknown new TF[17]	Regulates expression of tyrosine kinase being essential in monocytic and neutrophilic development[17, 18]	504*
mrp-8	IRF-1, CDP/NF-y, CBP, GT1, PU.1[19]	Regulates expression of myeloid calcium binding protein MRP-8 found in granulocytes, monocytes and macrophages[20], which is involved in regulation of NADPH-oxidase.	1584*
sp-107	PU.1, SP1 sites, c/EBPα, AML[21]	Synthetic promoter element generated by random ligation of myeloid/macrophage cis elements[21].	265*
microRNA-223 (miRNA-223)	Not yet published; e.g. PU.1, ISRE, Evi1 (predicted by MatInspector (Genomatix, software)).	Involved in regulation of granulocyte differentiation and linked miR-223 RNA showed a highly lineage-specific expression pattern for miR-223[22].	783*

Table 1 Short description of myelospecific promoters used in this study. Abbreviations: purine rich box-1 (PU.1); interferon-stimulated responsive elements (ISRE); high mobility group (HMG)-box containing protein 1 (HBP1); polyoma virus enhancer activator 3 (PEA3); phosphatidylethanolamine binding protein 2 (PEBP2); interferon regulatory factor 1 (IRF-1); CCAAT-box binding TF (NF-Y CDP/NF-y CBP GT1); TF erythroblastosis virus E26 oncogene homologue 1 (Ets-1); CREB-binding protein (CBP); C/AAT enhancer binding protein (c/EBPα); acute myeloid leukemia-1 (AML); ecotropic viral integration site-1 (Evi1); *refers to size of the specified promoter-element used in this study.

Gene expression relies not solely on gene-proximal elements such as promoters, enhancers and silencers. Long-range interactions of various cis regulatory elements and chromatin modifications can also influence gene expression. This was shown e.g. for the locus control regions (LCR) which have the ability to enhance the expression of linked genes in a tissue-specific and copy number-dependent manner at ectopic chromatin sites[23]. For the *cfes* gene, a LCR was identified which in combination with the myelospecific cfes promoter directed transgene expression in a myeloid-restricted manner[24].

To develop a SIN vector for $p47^{phox}$ CGD GT we performed two rounds of screening to find a potent internal promoter. In the first screen, different myelospecific promoters driving GFP expression were tested. Potent candidates were then cloned into a (γ)-retroviral SIN vector 5' to the $p47^{phox}$ open reading frame (ORF). The constructs were screened for *ex vivo* myelospecific expression of $p47^{phox}$ and functional reconstitution in transduced murine $p47^{phox}$ -/- HSCs upon propagation to granulocytes in cell culture. These two screens revealed a new potent promoter, miRNA-223, which restricts $p47^{phox}$ expression exclusively to granulocytes.

Results

First screen for myelospecificity in PLB-985 cells

In a first screen a panel of (mainly human p47^{phox}-promotor based) lentiviral promoter constructs was tested for myelospecific expression of green fluorescent protein (GFP) as a marker gene (figure 1). We tested these constructs in premyeloid PLB-985 cells (DSMZ no.: ACC 139) as these cells can be differentiated to neutrophils *in vitro*.

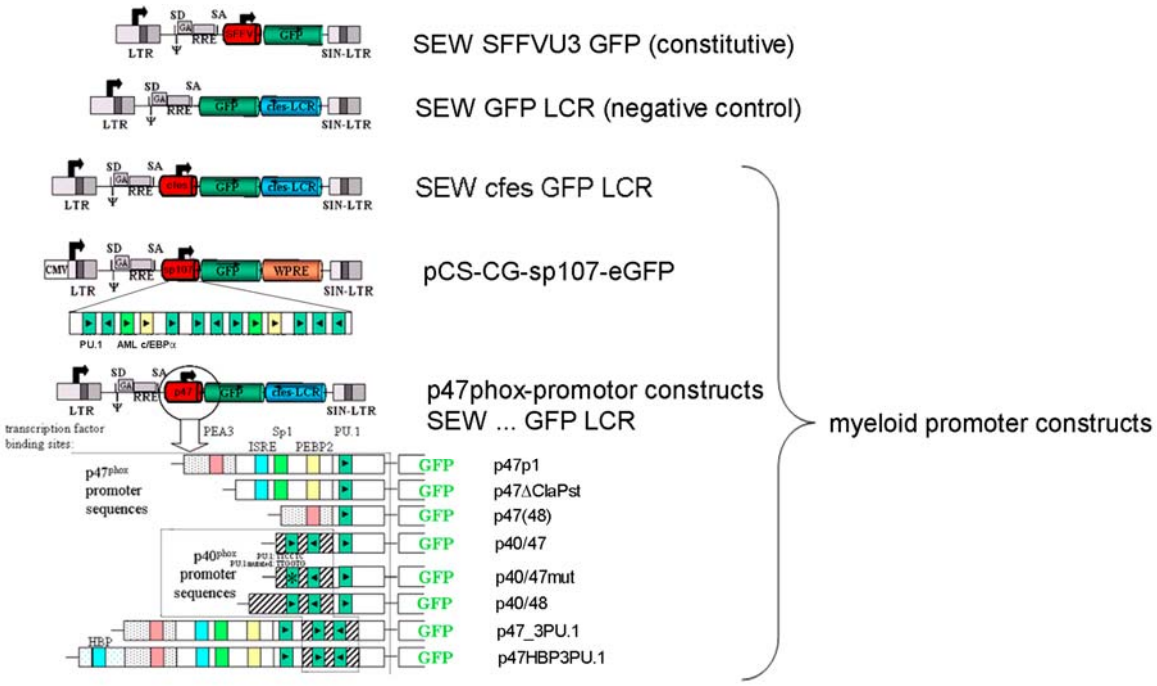

Figure 1 Schematic diagram of the vectors used in the first screen. The structures of the cloned lentiviral vector plasmids are shown.
SEW SFFVU3 GFP is a lentiviral SIN vector (majority of U3 deleted in the 3'-LTR with an internal SFFV promoter) driving GFP-expression and used as constitutive active positive control. SEW GFP LCR is the negative control without promoter.
SFFV denotes the enhancer and promoter elements derived from the LTR of the spleen focus forming virus. Ψ, packaging signal; LTR, long terminal repeat; SA, splice acceptor site; SD, splice donor site; RRE, rev-responsive element; GA, truncated *gag* region; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; cfes-LCR, cfes-locus control regions;

For each promoter construct, virus supernatant was produced by calcium phosphate mediated cotransfection of human embryonic kidney (HEK) 293T cells (ACC 305) with three plasmids: pCMVΔ8.91 (encoding the lentiviral Gag/Pol), pMDG2.G (encoding the VSV envelope (vesicular stomatitis virus G glycoprotein)) and with the gene transfer vector as shown above (figure 1).

To quantify the myelospecificity of our promoter constructs, myeloid cell line PLB-985 cells were transduced at molarity of infectivity (MOI) equal to 1 according to prior titration. Thereafter, half of each transduced cell population was propagated to terminally differentiated neutrophils by liquid cell culture in presence of 0.5% dimethylformamide (DMF) for 6 days. The other half of the transduced cells was cultured further in parallel. Both transduced cell populations, the undifferentiated PLB-985 cells and the CD11b+ PLB-985 cells, were then analyzed for GFP expression by flow cytometry.

The absolute GFP signal intensity in differentiated PLB-985 neutrophils (CD11b+) (figure 2b) as well as the ratio between the means of differentiated and undifferentiated PLB-985 cells (figure 2a) were highest for the synthetic promoter construct “sp107” (with nine PU.1 transcription factor binding sites) followed by the mixed promoter “p40/48“ (with three PU.1 sites) indicating myelospecificity and inducibility of these two promoters.

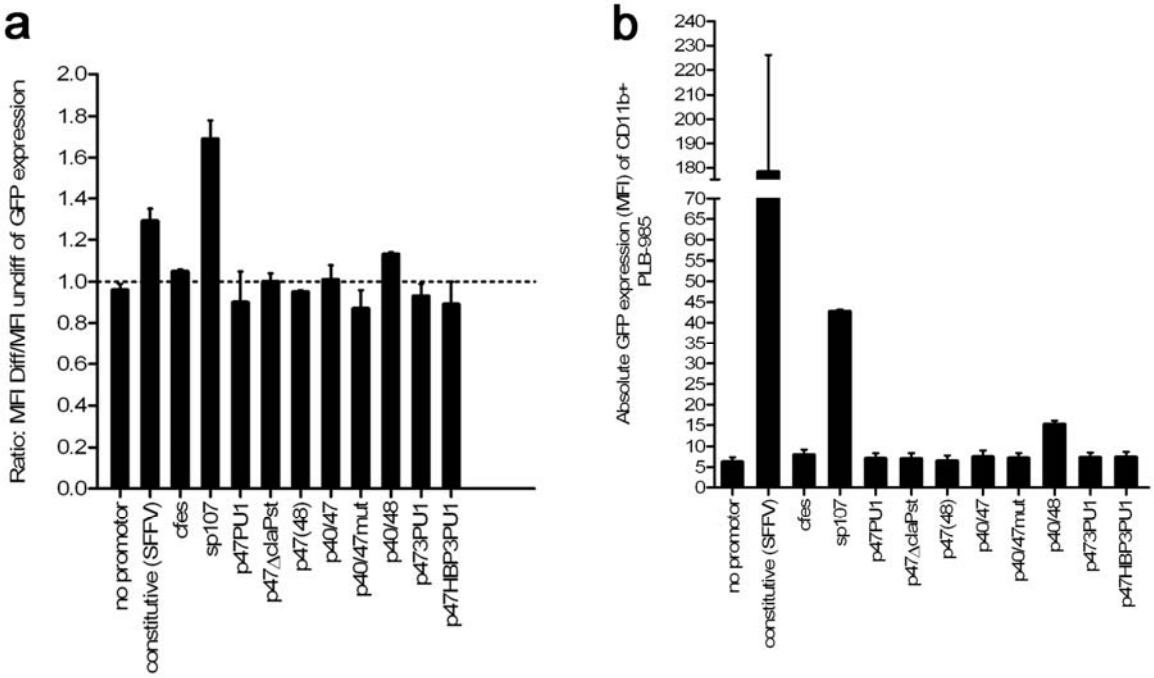

Figure 2 Screen of myelospecific GFP expressing lentiviral promoter constructs in premyeloid PLB-985 cell line. PLB-985 cells were lentivirally transduced. PLB-985 cells were differentiated in liquid cell culture in the presence of 0.5% DMF. The expression of GFP was monitored by FACS analysis after 6 days in undifferentiated and in differentiated CD11b+ PLB-985 cells.
(**a**) Ratio of GFP mean fluorescence intensity (MFI) between CD11b+ (differentiated) and undifferentiated PLB-985 cells transduced with indicated lentiviral SIN vectors. Means and standard deviations of two independent experiments are shown. To avoid multicopy integrations, transduction rates were kept at MOI=1 according to prior performed titration.
(**b**) Absolute mean GFP fluorescence intensity (MFI) within the GFP-positive population of differentiated CD11b+ PLB-985 cells. Means and standard deviations of two independent experiments are shown. To avoid multicopy integrations, transduction rates were kept at MOI=1 according to prior performed titration.

Generation of gammaretroviral SIN vector constructs

This first screen allowed us to pre-select the most promising candidates among the tested promoter constructs according to their myelospecific GFP expression in the premyeloid PLB-985 cell-line[25]. In the next step, we cloned the p47^{phox} transgene (M25665, codon optimized) into a γ-retroviral pSERS11M8delN91s SIN vector (kind gift of M. Grez, Frankfurt, Germany) to express p47^{phox} in hematopoietic cells. This vector contains an optimized backbone architecture including deletion of enhancer and TATA box (SIN configuration) and an internal myelospecific mrp8 promotor[20]. Based on this construct we generated retroviral constructs containing the p47^{phox} transgene under control of different internal myelospecific promoters: sp-107, cfes, p40/48, miRNA-223 (miR-223) and for comparison the constitutively active SFFV promoter (figure 3).

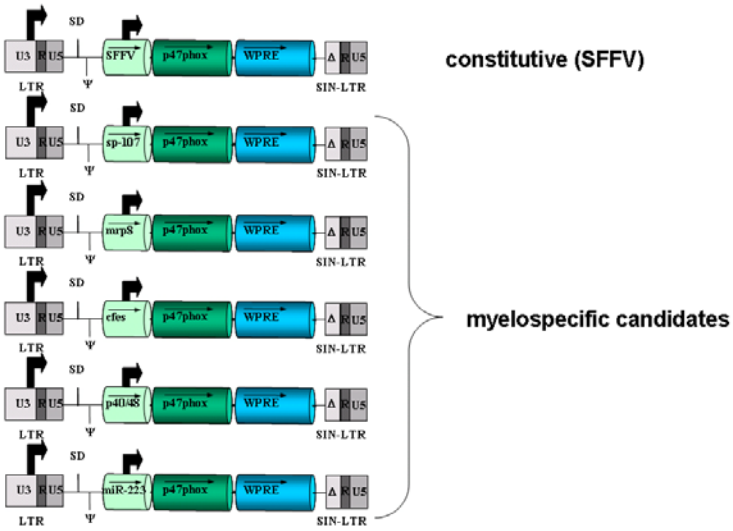

Figure 3 Schematic diagram of cloned gamma (γ) -retroviral SIN vectors with p47^{phox} as transgene (plasmid configuration) under control of different internal myelospecific promoters. The SFFV promoter construct was used as constitutive active control.
The following items are indicated: The SIN configuration is indicated by partial deletion of the U3 (Δ) in the 3' LTR and the presence of an internal promoter. SD, splice donor site; Ψ, packaging signal; LTR, long terminal repeat. WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; U3, R, U5 denote LTR subunits.

Screen of myelospecific promoter constructs in primary murine p47^{phox} -/- lineage negative bone marrow cells

To select the most promising promoter candidate we transduced murine p47^{phox} -/- lineage negative (Lin-) bone marrow (BM) cells with the generated γ -retroviral vectors expressing p47^{phox} under control of different myelospecific promoters at low MOI. 48h after transduction, the cell populations were split in two. One half was analyzed for p47^{phox} transgene expression in undifferentiated ScaI+ cells. The other half was *ex vivo* differentiated to Ly6G (GR1)+ granulocytes in liquid cell culture for at least 10 days followed by p47^{phox} flow cytometry analysis (FACS). In ScaI+ undifferentiated cells the transduction efficiency was below 25% confirming that the majority of the transduced cells contained statistically just one integration per cell[26]. Among all tested myelospecific constructs the miR-223 promoter showed the highest p47^{phox} mean fluorescence intensity (MFI) within the Ly6G (Gr1)+ population, followed by the p40/48 promoter and the cfes promoter constructs (figure 4c). Analysis of the p47^{phox} MFI ratio between differentiated (Ly6G+/p47^{phox}+) and undifferentiated (ScaI+/p47^{phox}+) cells lead to the same conclusion. Among all promoter constructs miR-223 promoter construct showed most myelospecific p47^{phox} MFI expression compared to the constitutive active SFFV control (figure 4b).

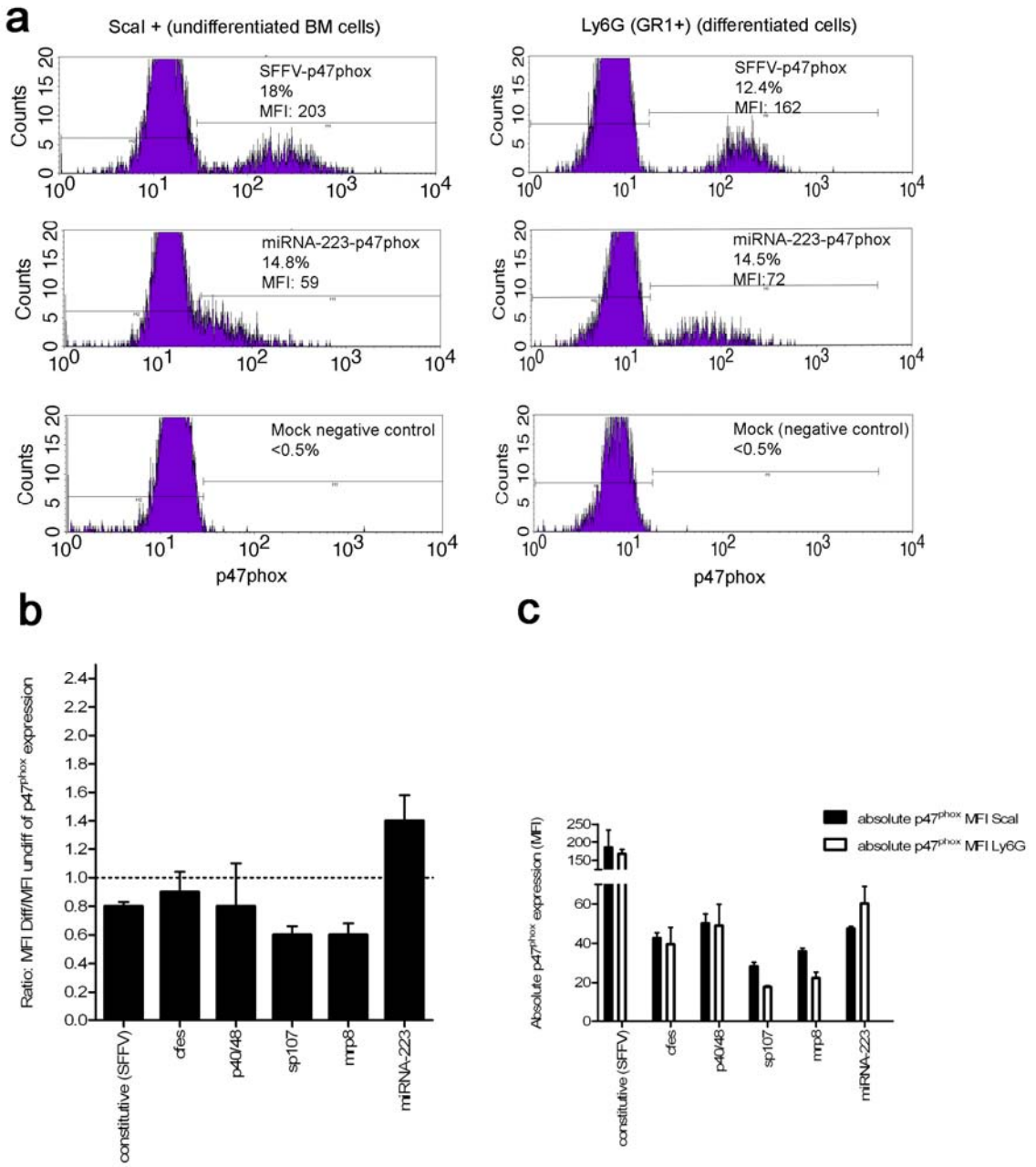

Figure 4 Screen of myelospecific p47^{phox} expressing promoter constructs in primary murine p47^{phox}-/- BM cells. Lin- BM cells of p47^{phox} -/- mice were retrovirally transduced. The expression of the p47^{phox} transgene was monitored by FACS analysis after 4 days in ScaI+ BM cells and in Ly6G+ (Gr1+) cells upon differentiation in liquid cell culture.
(**a**) Representative histograms of murine Lin- BM cells transduced with SFFV-p47^{phox} or miR-223-p47^{phox} γ-retroviral SIN vectors at low (MOI) in undifferentiated ScaI+ murine bone marrow (BM) cells and in differentiated Ly6G+ (GR1+) positive cells. To avoid multicopy integrations, transduction rates were MOI=1; γ-retroviral titers were predetermined in the murine pro B cell line BA/F3 (ACC 300) by real-time Q-PCR. MFI of the p47^{phox} positive cells was measured by performing intracellular staining of p47^{phox} followed by flow cytometry analysis.
(**b**) Induction of p47^{phox} transgene expression upon differentiation: Ratio of MFI in ScaI+ BM cells divided by MFI in GR1+ cells. MFI ratios and standard deviations of at least three independent experiments are shown.
(**c**) Absolute p47^{phox} expression (FACS MFI) in undifferentiated ScaI+ BM cells and in differentiated GR1+ cells. MFIs and standard deviations of at least three independent experiments are shown.

Functional reconstitution of NADPH oxidase activity in transduced and *ex vivo* differentiated Ly6G (Gr1)+ murine neutrophils

The production of reactive oxygen species (ROS) in granulocytes can be monitored by the dihydrorhodamine-123 (DHR) assay. In this assay, the oxidation of non-fluorescent DHR 123 to fluorescent rhodamine by ROS allows the detection of ROS producing cells by FACS analysis. Therefore we applied this methodology to analyze whether the vector constructs are able to restore the NADPH oxidase activity in Ly6G/Ly6C+ granulocytes. Upon phorbol 12-myristate 13-acetate (PMA) stimulation, the shift in DHR signal (figure 5a) demonstrated that transduction by the constitutive control (SFFV) or the myelospecific promoters (cfes, p40/48 and miRNA-223) constructs resulted in the restoration of NADPH oxidase activity. The percentage of 5 to 21% DHR+ cells reflects the expected transduction rate after transduction with MOI=1. Only a small fraction (about 0.5%) of DHR+ cells could be observed in case of the sp107 and mrp8 promoter constructs (figure 2b) which resulted from significant lower virus titers. Analysis of the MFI within the DHR+ cell population showed similar shift between the constitutive control and the indicated myelospecific promoter constructs (figure 2c). Taken together, we have shown in our screen that the miRNA-223 promoter construct showed the best induction of $p47^{phox}$ expression upon neutrophilic differentiation among all other promoter candidates and that the miRNA-223 promoter construct restored the NADPH oxidase activity in murine $p47^{phox}$ -/- cells.

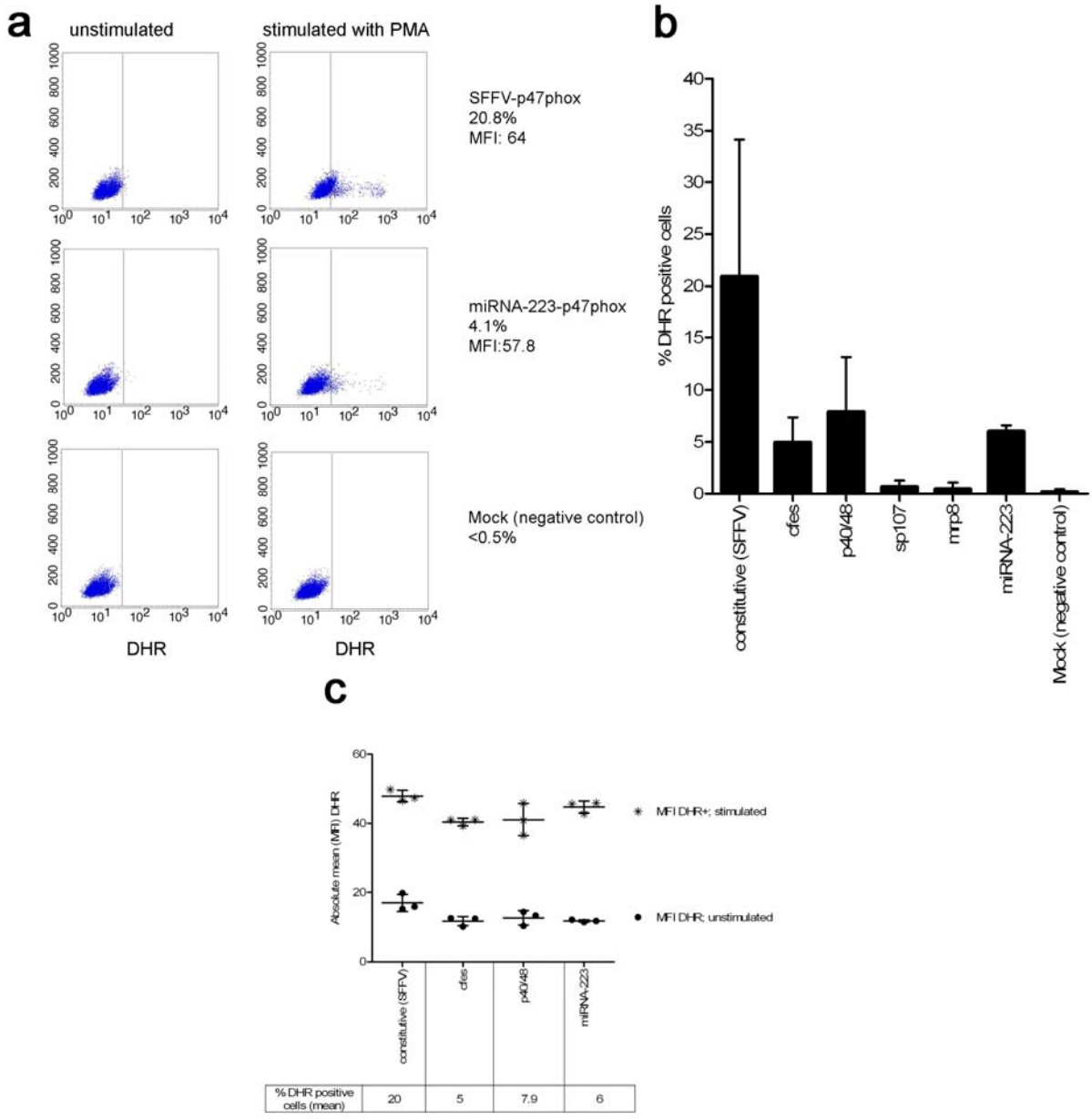

Figure 5 γ-retroviral reconstitution of the NADPH oxidase activity. Murine Lin- BM cells from p47^{phox} -/- mice were γ-retrovirally transduced and differentiated to Ly6G (Gr1)+ granulocytes. The reconstitution of NADPH oxidase activity upon PMA stimulation was analyzed by a DHR 123 oxidation assay within the Gr1+ cell population.
(**a**) Representative examples of FACS based DHR 123 oxidation analysis in Gr1+ cells. % and MFI of DHR positive population within Ly6G (Gr1)+ murine neutrophils are indicated.
(**b**) Percentage (%) of DHR+ cells within Ly6G (Gr1)+ murine neutrophils. % and standard deviations of three independent experiments are shown.
(**c**) MFI of the DHR+ population in stimulated and unstimulated granulocytes. MFIs and standard deviations of three independent experiments are shown.

Discussion

To develop a GT vector aimed to correct the p47^{phox}-deficient form of CGD we screened several promoter sequences for their potency of driving transgene expression in myeloid cells, especially in neutrophils. The rationale of this approach is based on the fact that the observed clonal dominance and malignant transformations resulted from the transactivation of oncogenes adjacent to the viral integration site in stem- and progenitorcells. Therefore, restricting the expression of therapeutic transgene to short-lived neutrophils will decrease the likelihood of side effects like malignant transformation.

We first screened our promoter candidates in PLB-985 cells. The differentiation status of the pre-myeloid cell line PLB-985 is somewhere in the middle on the differentiation pathway between HSCs and neutrophils. The cells can be differentiated *in vitro* either to granulocytes in the presence of DMF or dimethylsulfoxide (DMSO), or to monocytes in the presence of PMA[25]. The myelospecificity of our promoters was estimated according to the increase in promoter candidates' driven GFP expression upon differentiation to granulocytes. Surprisingly, only two constructs showed induction of GFP expression upon myelospecific differentiation. These were namely the synthetic promoter construct "sp107" and the mixed "p40/48" promoter consisting of human p47^{phox} and p40^{phox} promoter sequences. The other constructs mainly based on the human p47^{phox} promoter. It is not clear, why these constructs did not show myelospecific expression of GFP. Possibly, these p47^{phox} promoter constructs are already activated to a certain extent on the differentiation status of undifferentiated PLB-985 cells. Hence, differentiation would result in only a weak activation of these promoters in the PLB system. Among the p47^{phox} promoter constructs the mixed promoter "p40/48" showed the strongest increase in GFP expression upon myelospecific differentiation. The "p40/48" promoter consists of p47^{phox} and of p40^{phox} sequences and possesses all together three PU.1 sites. The synthetic promoter sp107 contains nine PU.1 sites and showed an even stronger myelospecific GFP expression. These results are in line with the reported role of TF PU.1 in myelospecific expression[11, 15, 16, 27]. However, the use of the sp107 vector with nine repetitive PU.1 sites needs to be analyzed carefully. It is known that repetitive sequences within retroviral genomes are unstable and are deleted frequently upon reverse transcription; a process called reverse transcriptase (RT) template switching[28, 29]. However, none of the other cloned TF binding sites contributed as strong as the PU.1 TF site to myelospecific expression. They rather seem to repress myelospecific GFP expression as seen for the construct "p47_3PU.1".

In summary, this first screen in PLB-985 cells revealed a moderate myelospecific property for the cfes promoter and significant myelospecific transgene induction by the "p40/48" and the "sp107" promoter.

We then switched to γ-retroviral vectors containing the p47^{phox} transgene instead of GFP reporter gene, since γ-retroviral vectors have been used in the latest temporarily successful clinical GT X-CGD trial[7].

For detection of the cytoplasmic p47^{phox} transgene product we established the intracellular staining (ICS) with an anti-p47^{phox} antibody followed by flow cytometry analyses. The detection of p47^{phox} by ICS is new as in previous p47^{phox} studies the expression was mainly detected by western blot analyses of crude cell extracts[30] or indirectly by performing functional NADPH oxidase activity assays[5]. Furthermore, we increased the γ-retroviral vector titers by two logs and we significantly reduced their toxicity. This was done by (I) optimizing the plasmid concentration utilized for virus production (II) shifting from the calcium phosphate method to a transfection reagent and (III) concentrating the virus supernatant.

The 2nd screen was performed in primary murine p47^{phox} -/- Lin- BM cells; the primary target organ in later clinical use. In addition to the three promoters favoured by the first screen, we tested the “mrp8” promoter and the “miRNA-223” promoter (kind gift of our collaboration partner M. Grez). Again, myelospecificity was estimated by the ratio between the expression in Lin- BM cells and in terminally differentiated Gr1+ granulocytes.

In case of the synthetic promoter “sp107” the myelospecific expression seen in the GFP PLB-985 screen could not be confirmed in the 2nd screen. In the case of the “mrp8” promoter construct it was difficult to obtain high titers. This could be due to the promoter size (around 1.5 kb). Furthermore, the “mrp8” promoter contains splice sites which might result in deletions during retroviral life cycle.

By far the strongest induction of p47^{phox} expression was observed for the “miRNA-223” promoter. Considering the absolute MFI values of p47^{phox} expression, a relatively high signal in undifferentiated ScaI+ cells of all constructs was observed. This was unexpected for myelospecific promoters as they should not be active in the progenitor/stem cells. Possibly, the high MFI of p47^{phox} expression measured in undifferentiated ScaI+ cells is due to the presence of unintegrated circular DNA[31]. In terminally differentiated granulocytes these unintegrated circular viral DNA got lost due to dilution as the cells proliferate upon differentiation. We determined the MOI in terminally differentiated granulocytes. This MOI just refers to the integrated viral DNA. The actual MOI which results initially in unintegrated circular DNA plus integrated DNA might be higher. Therefore, we can’t exclude the possibility that we compared in our assay multiple copies of unintegrated circular DNA in progenitor/stem cells with a single copy situation in granulocytes. This challenge is hard to circumvent as later analysis of undifferentiated ScaI+ cells bears the risk of loosing undifferentiated cells due to continuous differentiation. If this is true, minor myelospecific expression effects can not be detected by this screen. This would possibly explain the absence of myelospecificity observed in other myelospecific promoter constructs.

Finally, we demonstrated in p47^{phox} -/- cells that the transgenic expression of p47^{phox} under control of “cfes”, “p40/48” or the “miRNA-223” promoter resulted in reconstitution the NADPH oxidase function. For all promoter constructs the shift in the FACS signal within the DHR test was comparable to the shift observed for the strong and constitutively active SFFV promoter. Already 5% of functional granulocytes are sufficient to protect a patient against infections[32-34]. Therefore GT with myelospecific SIN vectors as they were used in this study could lead to sufficient number of corrected cells with sufficient NADPH oxidase activity. The presented combination of a γ-retroviral SIN vector with a strictly myelospecific internal promoter, e. g. miRNA-223, represents a major safety improvement as it minimizes the risk of malignant transformations in stem- and progenitor cells as longterm effect of insertional mutagenesis.

Materials and methods

Cell culture

Non-adherent growing PLB-985 (ACC 139) cells were grown in RPMI 1640 (PAA Laborotories), 2% Antibiotic/Antimycotic (PAA Laborotories). PLB-985 cells were differentiated to neutrophils in RPMI 1640 (PAA Laborotories), 0.5% DMF (Sigma), 2.5% foetal bovine serum (FBS) (PAA Laborotories), 2% Antibiotic/Antimycotic (PAA Laborotories) for 6 days. Murine pro B cell line BA/F3 (ACC 300) were cultured as were PLB-985 cells supplemented with 10ng/ml mIL-3 (Peprotech).

Murine $p47^{phox}$ -/- Lin- BM cells were harvested from femora and tibiae of $p47^{phox}$ -/- mice (B6(Cg)-Ncf1m1J/J) (Jackson Laboratory) and enriched by the "Lineage Cell Depletion Kit" (Miltenyi Biotec). Isolated BM cells were cultured in StemSpan H3000 medium (StemCell Technologies) supplemented with 50 ng/ml murine stem cell factor (mSCF) (Peprotech), 100 ng/ml human Flt-3 ligand (Peprotech), 100 ng/ml human interleukin-11 (IL-11) (Peprotech), 20 ng/ml murine IL-3 (Peprotech), 1% penicillin/streptomycin (GIBCO), 2 mM glutamine (GIBCO)[35] and 2% FBS (Hyclone Thermoscientific).

For differentiation of murine Lin- BM cells to granulocytes see section "Transduction of murine $p47^{phox}$ -/- Lin- BM cells".

Cloning of lentiviral vectors and production of infectious particles

For SIN lentiviral vectors, the backbone was described recently[36]. The internal promoter fragment (EcoRI/BamHI) was exchanged by PCR products of different myelospecific promoter sequences as illustrated above (figure 1). cfes-LCR was cloned into lentiviral vector into its MluI site. Infectious particles were transiently generated by HEK 293T cells (ACC 305) by transfecting a 70-80% confluent cell layer using calcium phosphate methodology. In a 10 cm petridish 6.5μg pCMVΔ8.91 (encoding lentiviral GagPol), 3.5μg pMDG2.G (encoding VSV envelope) and 10μg gene transfer vector were co-transfected into HEK 293T packaging cells. Cells were incubated for 6h in the presence of the DNA/calcium phosphate precipitate and 100μM Chloroquin (Sigma), washed and cultured in fresh medium. Infectious particles containing cell culture supernatants were collected 24h and 48h after transfection, sterile filtered (Millipore) and stored immediately at -80°C. For titration, serial dilutions of viral supernatants were utilized to infect PLB-985 cells (ACC 139) in the presence of protamine sulfate (8μg/ml, Sigma). Freshly thawed virus supernatant was spinoculated onto the cells (90 min, 2500 RPM, at 32°C). 3 days later the percentage of green fluorescent protein (GFP) expressing cells was determined by flow cytometry (FACS Calibur, Becton-Dickinson (BD) Biosciences). Transduction efficiencies above 20 % result in a significant portion of cells with

more than one integration[26]. Therefore, we calculated the titer just from those transductions which resulted in a maximum of 20% GFP+ cells. For calculation we used following formula:

Titer (transducing units/ml) =
= seeded cell number x dilution factor of virus supernatant x (GFP positive cells (%)/100%).

Cloning of γ-retroviral vectors and production of infectious particles

The γ-retroviral pSERS11M8delN91s SIN vector (a derivate of the SERS11.SF.GFP.W [10], kindly provided by M. Grez, Georg Speyer, Frankfurt) was used as basic construct. pSERS11M8delN91s SIN vector contains the internal mrp8 promoter 5' to the $gp91^{phox}$ coding reading frame (CRF). In the first step a Sal I-restriction site was inserted between the $gp91^{phox}$ CRF and the Woodchuck hepatitis virus post-transcriptional element (WPRE) by site directed mutagenesis (Stratagene). Then, the $gp91^{phox}$ CRF was substituted by human $p47^{phox}$ CRF (M25665, codon optimized by GENEART) by BamH1/SalI (New England Biolabs (NEB)) restriction digestion. All other promoters were cloned into this vector by replacing the NdeI/BamHI mrp8 promoter fragment. The constitutive active promoter sequence SFFV was amplified from SERS11.SF.GFP.W (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany). The sp107 promoter derived from "Lenti-sp107-GFP" [21] (kindly provided from Senlin Li, University of Texas, Department of Medicine, USA) and the miRNA-223 promoter from "223-1 gps W" (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany).

For construction, the recombinant plasmids were introduced by electroporation (Gene Pulser, Biorad) or heat shock into E. Coli Top-10 competent cells (Invitrogen). DNA manipulations were performed according to standard procedures by using commercial kits (Quiagen, Macherey-Nagel, Genomed). All resulting constructs were controled by restriction digestion followed by agarose gel electrophoresis. The cloning sites and flanking sequences of the resulting constructs were confirmed by DNA sequencing (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems (AB)).

Ecotropic infectious particles were generated by transient transfection of Phoenix (ΦE) cells (ACC 3444). These cells are derived from HEK 293T cells by stably integration of a plasmid carrying the moloney murine like virus (MLV) *gag-pol* CRFs and with an ecotropic MLV *env* expressing plasmid. In a 10 cm petridish, 70-80% confluent Phoenix E cell layers were co-transfected by 4μl TransIT-293 reagent (Mirus LCC Bio) per μg (total DNA), 10μg pUMVC (encoding γ-retroviral GagPol), 4μg M187 (encoding ecotropic envelope) and 40μg gene transfer vector. Infectious particle containing cell culture supernatants were collected 24h,

48h, 60h after transfection and sterile filtered (Millex Syringe Driven Filter units, 0.45 μm pore size (Millipore)). The supernatant was concentrated (30X) (Amicon-Ultra 15 centrifugal units, 1500g, 18min at RT) from 15ml to an end volume of 500μl and frozen immediately in form of aliquots a 20μl at -80°C. For titration, serial dilutions of viral supernatants were used to infect the murine pro B cell line BA/F3 (ACC 300) in the presence of protamine sulfate (8μg/ml, Sigma). Virus supernatant was thawed and taken immediately for transduction (spinoculation for 90 min, 2500 RPM, at 32°C). One week after transduction DNA was extracted from the cell pellets by „DNeasy blood and tissue kit" (Qiagen). The vector copy number was quantified by real-time quantitative PCR (q-PCR) with 50-200 ng template DNA in a reaction volume of 25μl. The set of primers and probes used for the analysis were as follows (table 2):

detect	primers/probes	sequence	final concentration, nM	company
actb (encoding β-actin) (endogenous control)	forward primer	5'-AGAGGGAAATCGTGCGTGAC-3'	500	Microsynth
	reverse primer	5'-CAATAGTGATGACCTGGCCGT-3'	500	Microsynth
	probe	VIC-CACTGCCGCATCCTCTTCCTCCC-MGB	200	AB
WPRE	forward primer	5'-TTTCTGGGACTTTCGCTTTCC-3'	500	Microsynth
	reverse primer	5'-AGGCGGCGATGAGTTCTG-3'	500	Microsynth
	probe	FAM-CCTCCCGATCGCC-MGB	200	AB

Table 2 Primers and probes used for real-time q-PCR.

We generated a standard curve by combining DNA from BA/F3 cells without and with one integration per cell (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany). TaqMan reactions were carried out in triplicates in an ABI Prism 7700 HT Sequence Detection System (AB). Transduction of HSC and different cell lines (e.g. BA/F3 cells) with one and the same virus supernatant resulted in different transduction efficiencies. Therefore, we determined the factor between the titers related to BA/F3 cells and HSC. Murine Lin- BM cells and BA/F3 cells were transduced with the SFFV control virus supernatant and analyzed 5 days later by intracellular $p47^{phox}$ staining measured by FACS (BD). The titer in BA/F3 cells was calculated to be 1.6 fold higher compared to HSCs.

Detection of transgene expression in individual hematopoietic cell populations

In differentiated PLB-985 cells granulocytic maturation was estimated according to CD11b induction. Only CD11b+ cells (antibody-clone ICRF44) were analyzed for GFP expression.

p47^{phox} expression was measured by performing ICS with the anti-p47^{phox} antibody (clone 1), APC-labeled by BD-services. For combined staining of surface markers and intracellular p47^{phox}, the cells were first blocked with mouse Fc-block (BD) (clone 2.462) for 10min at 4°C, incubated with the surface antibodies for 1h at 37°C and washed twice. Thereafter, p47^{phox} was stained with APC anti-p47^{phox} using the Cytofix/Cytoperm Kit (BD) according to manufacturer's instructions. FITC anti-Ly-6G (clone 1A8, BD) was used to detect murine neutrophils and anti-Ly-6A/E ScaI (clone E13-161.7, BD) to detect murine stem- and progenitor cell enriched cell populations.

Transduction of murine p47^{phox} -/- Lin- BM cells

p47^{phox} -/- Lin- BM cells were isolated using the lineage cell depletion kit (Miltenyi). The BM cells were cultured for 2 to 3 days in StemSpan H3000 supplemented with cytokines (see cell culture section). Cell culture plates were precoated with Retronectin (TaKaRa) by incubation with 10 μg/cm^2 at RT for 2h followed by HSA incubation for 10 min. Frozen titered γ-retroviral supernatants were thawed, prediluted in StemSpan H3000 and loaded onto the precoated plates by centrifugation (3000 RPM for 30 min at 4°C). Then, p47^{phox} -/- Lin- BM cells were seeded onto virus-loaded plates and cultured for 3 to 5 days in cytokine supplemented StemSpan H3000[35].

For later intended p47^{phox} FACS analysis of *ex vivo* differentiated granulocytes, the Lin- BM cells were washed two days after transduction and incubated for 10-12 days in fresh stem cell culture medium additionally supplemented with 100ng/ml mG-CSF.

For later intended functional analysis (DHR assay) of *ex vivo* differentiated granulocytes, the Lin- BM cells were washed twice 2-3 days after transduction to get rid off all "stem cell cytokines". The cells were then incubated in RPMI, 20% FBS, 100ng/ml mG-CSF and 10 ng/ml mIL-3 for 4 to 6 days.

Analysis of respiratory burst activity

For analyses of respiratory burst activity, the dihydrorhodamine assay (DHR) 123 was carried out as described[7] with one modification. We combined the FACS based DHR assay with the analysis of cell surface markers. In the DHR 123 assay, *ex vivo* differentiated murine neutrophils (see above) were identified by prestaining with PerCP-Cy5.5 anti-Ly-6G and Ly-6C (clone RB6-8C5, BD). Then, the cells were stimulated with 1μg/ml PMA (SIGMA) for 15min at 37°C in presence of DHR. A shift in DHR 123 fluorescence was measured by flow cytometry (BD) within 30 min after stimulation.

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Chapter 2:
Gene therapy of p47^{phox} -/- deficient CGD mice using new myelospecific self-inactivating gammaretroviral vectors

written as manuscript for publication

Contributions: All experiments were conducted by Vital Wohlgensinger in Zürich. Vital Wohlgensinger and Ulrich Siler analyzed the results. All figures in this chapter were designed by Vital Wohlgensinger. Ulrich Siler designed the research.

Abstract

First clinical success in retroviral GT could be achieved for X-CGD and ADA-SCID, but were accompanied with clonal dominance or leukemic progression as long term effects of transactivation events. To develop a GT vector for the p47^{phox}-deficient form of CGD we have previously screened various myelospecific promoters in gamma (γ) -retroviral self-inactivating (SIN) vectors driving p47^{phox} expression. The best promoter candidate, the microRNA-223 (miRNA-223) promoter, was utilized in an *in vivo* GT in p47^{phox} -/- mice and the outcome was compared to the constitutive active control vector. GT with miRNA-223 vector resulted in clear myelospecific p47^{phox} transgene expression and restored the function of the NADPH oxidase in target neutrophils *in vivo*. The presented combination of a γ-retroviral SIN vector with a strictly myelospecific promoter represents a major safety improvement in CGD GT.

Introduction

Chronic granulomatous disease (CGD) results from an impaired respiratory burst activity caused by an inactive phagocyte NADPH oxidase complex[1]. This enzyme complex consists of two membrane spanning subunits (gp91^{phox} and p22^{phox}) plus three cytosolic components (p47^{phox}, p67^{phox} and p40^{phox}). Approximately 60% of all CGD cases were estimated to result from mutations in the X-linked *cybb*-gene encoding gp91^{phox} followed by additional 30% caused by mutations in the *ncf1*-gene encoding p47^{phox} [2]. Unfortunately, hematopoietic stem cell transplantation (HSCT) for CGD has been associated with unacceptably high rates of morbidity, mortality and graft failure, except in very selected cases in which a human leukocyte antigen (HLA) -identical donor is available[3, 4]. As a HLA-identical donor can only be found for about 50% of the patients[5] a therapy for CGD complementary to HSCT is highly desired for the other half of the patients lacking an appropriate BM donor. CGD could be successfully corrected in animal models by GT[6, 7]. Though limited in time, clinical success could be shown in recent GT aimed to correct X-CGD[8]. However, oncogene transactivation by viral LTR sequences resulted in clonal dominance[8] or leukemic progression[9]. These adverse events highlighted the risks associated with integrating viruses as vectors for gene delivery[10]. To minimize the risks of transactivation, we have previously developed series of SIN γ-retroviral vectors for p47^{phox} GT (see chapter 1). SIN vectors contain a deletion within the U3 region of the viral 3' LTR which results in inactivation of the viral promoter/enhancer elements upon reverse transcription[11]. Transgene expression is driven by an internal tissue-specific promoter element which ideally lacks enhancer activity. E. g. a myelospecific promoter restricts the transgene expression to terminally differentiated granulocytes/monocytes and thereby prevents transactivation events in HSC.

In our previous screen in *ex vivo* transduced murine p47^{phox} -/- hematopoietic stem cells (HSC), we have shown that the myelospecific microRNA-223 (miRNA-223) promoter induces the p47^{phox} expression upon granulocytic differentiation (see chapter 1). In the present study we conducted a BM transplantation in p47^{phox} -/- mice with *ex vivo* transduced (γ-SIN vector, miRNA-223 promoter, p47^{phox} transgene) autologous HSC to test its feasibility in p47^{phox} CGD GT. To validate the myelospecific p47^{phox} transgene expression, the outcome was compared to a GT with a control vector containing the strong constitutive SFFV promoter. We show here that GT in p47^{phox} -/- mice using miRNA-223 vector resulted in strictly myelospecific p47^{phox} transgene expression. Furthermore, GT with our vector in p47^{phox} -/- mice functionally corrected the CGD phenotype.

Results

(γ) -retroviral SIN vectors for GT of the p47^{phox} -deficient form of CGD

For GT of the p47^{phox} -deficient form of CGD we used two (γ) -retroviral SIN vectors which have been generated previously (see chapter 1). These vectors contain the human p47^{phox} transgene (M25665, codon optimized) under control either of the constitutive active SFFV promoter or of the myelospecific miRNA-223 promoter.

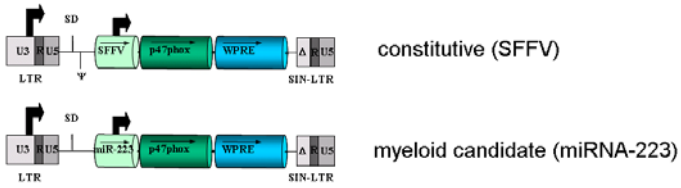

Figure 1 Schematic diagram of the used GT vectors for p47^{phox} -deficient form of CGD. The vectors are γ -retroviral SIN vectors (plasmid configuration) with p47^{phox} as transgene under control of the constitutive SFFV promoter or the myelospecific miRNA-223 promoter.
The following items are indicated: The SIN configuration is indicated by partial deletion of the U3 (Δ) in the 3' LTR and the presence of an internal promoter. SFFV, promoter/enhancer element of spleen focus forming virus; SD, splice donor site; Ψ, packaging signal; LTR, long terminal repeat; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element; U3, R, U5 denote LTR subunits.

GT of the p47^{phox} -deficient form of CGD in p47^{phox} -/- mice

GT of the p47^{phox} -deficient form of CGD was performed in p47^{phox} -/- mice[12]. Enriched murine p47^{phox} -/- lineage negative (Lin-) bone marrow (BM) cells were transduced *ex vivo* with one of the two γ-retroviral vectors shown above (figure 1) at molarity of infectivity (MOI) equal to 1 according to prior titration. 24h later, the transduced BM cells were transplanted into lethally irradiated p47^{phox} -/- mice (1E6 transduced BM cells per mouse).

Engrafted mice were killed six weeks post-transplantation. Blood, spleen and BM of each individual mouse were isolated. The p47^{phox} transgene expression in individual hematopoietic cell populations from each organ was detected by flow cytometry analysis (FACS) (see materials and methods). In Ly6G (GR1)+ granulocytes, p47^{phox} transgene expression could be detected in mice treated with the SFFV vector or the miRNA-223 vector (figure 2). Those p47^{phox} -/- mice which were treated with the myelospecific miRNA-223 SIN vector showed low or no p47^{phox} transgene expression in monocytes, B-lymphocytes, T-lymphocytes in contrast to the constitutive SFFV control mice. In the ScaI/CD117+ stem- and progenitor cells enriched population the p47^{phox} transgene expression was weak in the miRNA-223 mice group. The transduction efficiency of both vectors was below 25% confirming the majority of transduced cells contained statistically just one integration per cell[13].

Furthermore, splenic T-lymphocytes, B-lymphocytes and monocytes were analyzed on a genetic level to validate the presence of virus integrants. Though $p47^{phox}$ expression was hardly detectable in all three cell populations, quantitative real time PCR (q-PCR) analysis clearly revealed the presence of the transgene. This finding is in line with the myelospecificity of the miRNA-223 promoter and excludes the possibility that all transduced stem- and progenitor cells differentiated exclusively to granulocytes.

We further analyzed the absolute mean fluorescence intensity (MFI) of $p47^{phox}$ transgene expression in different hematopoietic cell populations to compare the transgene expression pattern of the two vectors. As expected, SFFV treated control mice showed a high absolute $p47^{phox}$ MFI in all hematopoietic cell populations measured (figure 3). A complete different result was obtained in case of the miRNA-223 group. These mice show a high $p47^{phox}$ MFI in blood and spleenic granulocytes, and a low or not detectable MFI in all other hematopoietic cell populations measured.

Furthermore, we judged granulocytic development according to their CD11b upregulation. In $CD11b^{low}$ granulocytic progenitors $p47^{phox}$ was undetectable whereas mature $CD11b^{high}$ granulocytes showed a high $p47^{phox}$ expression. As was true for granulocytic progenitors, $p47^{phox}$ expression was low or undetectable in all cell population analyzed but in mature granulocytes.

Altogether these results clearly show a myelospecific expression pattern of $p47^{phox}$ transgene in the miR-223 GT vector treated group.

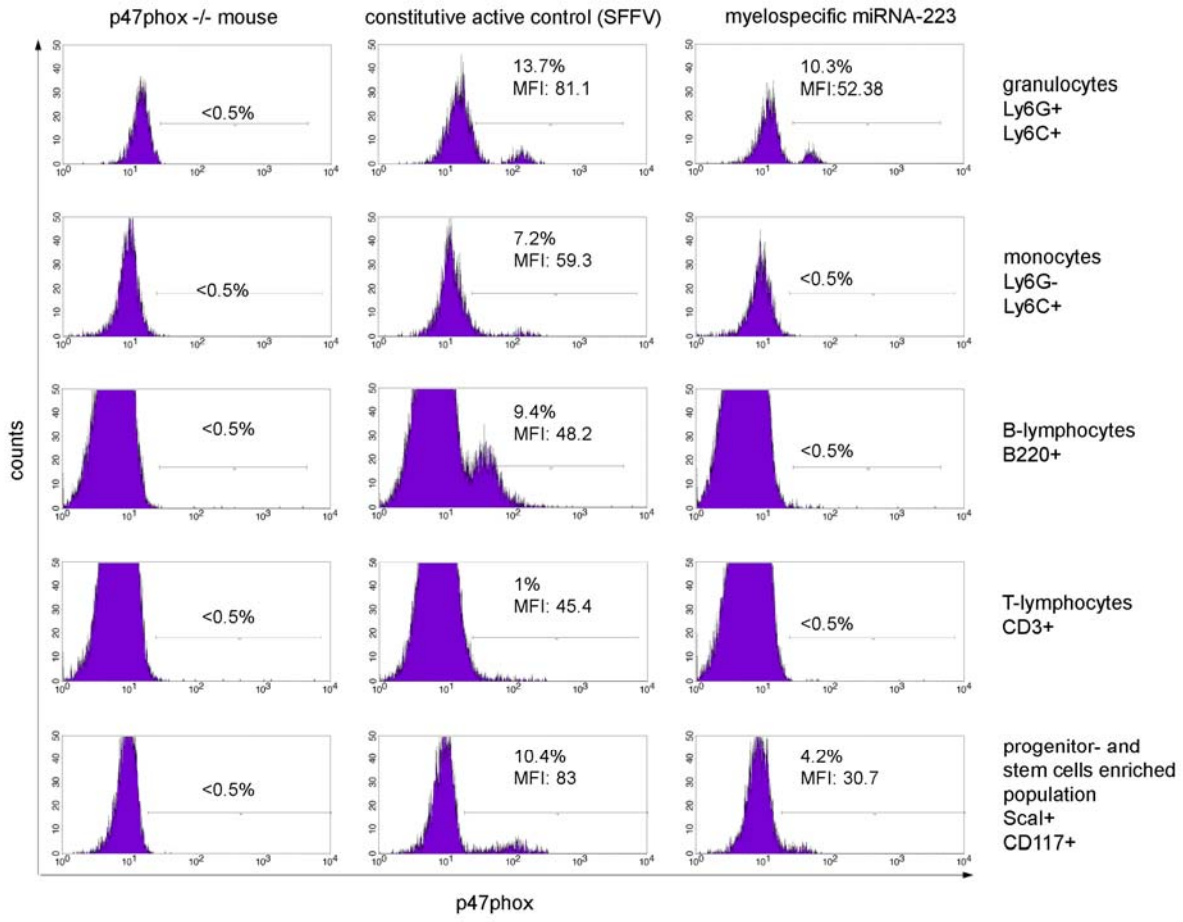

Figure 2 p47^{phox} transgene expression in p47^{phox} -/- mice after retroviral GT. Lin- BM cells of p47^{phox} -/- mice were *ex vivo* γ-retrovirally transduced and transplanted into lethally irradiated p47^{phox} -/- mice. 6 weeks after transplantation expression of p47^{phox} transgene was monitored in individual hematopoietic cell populations by FACS analysis.
Representative histograms of individual hematopoietic cell populations of blood and progenitor- and stemcells of BM from mice treated with SFFV-p47^{phox} or miR-223-p47^{phox} γ-retroviral vectors. Percentage (%) and mean fluorescence intensity (MFI) of p47^{phox} positive cells are indicated.
To avoid multicopy integrations, transduction rates were MOI=1; γ-retroviral titers were predetermined in the murine pro B cell line BA/F3 (ACC 300) by real-time q-PCR. MFI of the p47^{phox} positive cells was measured by performing intracellular staining (ICS) of p47^{phox}, followed by FACS.

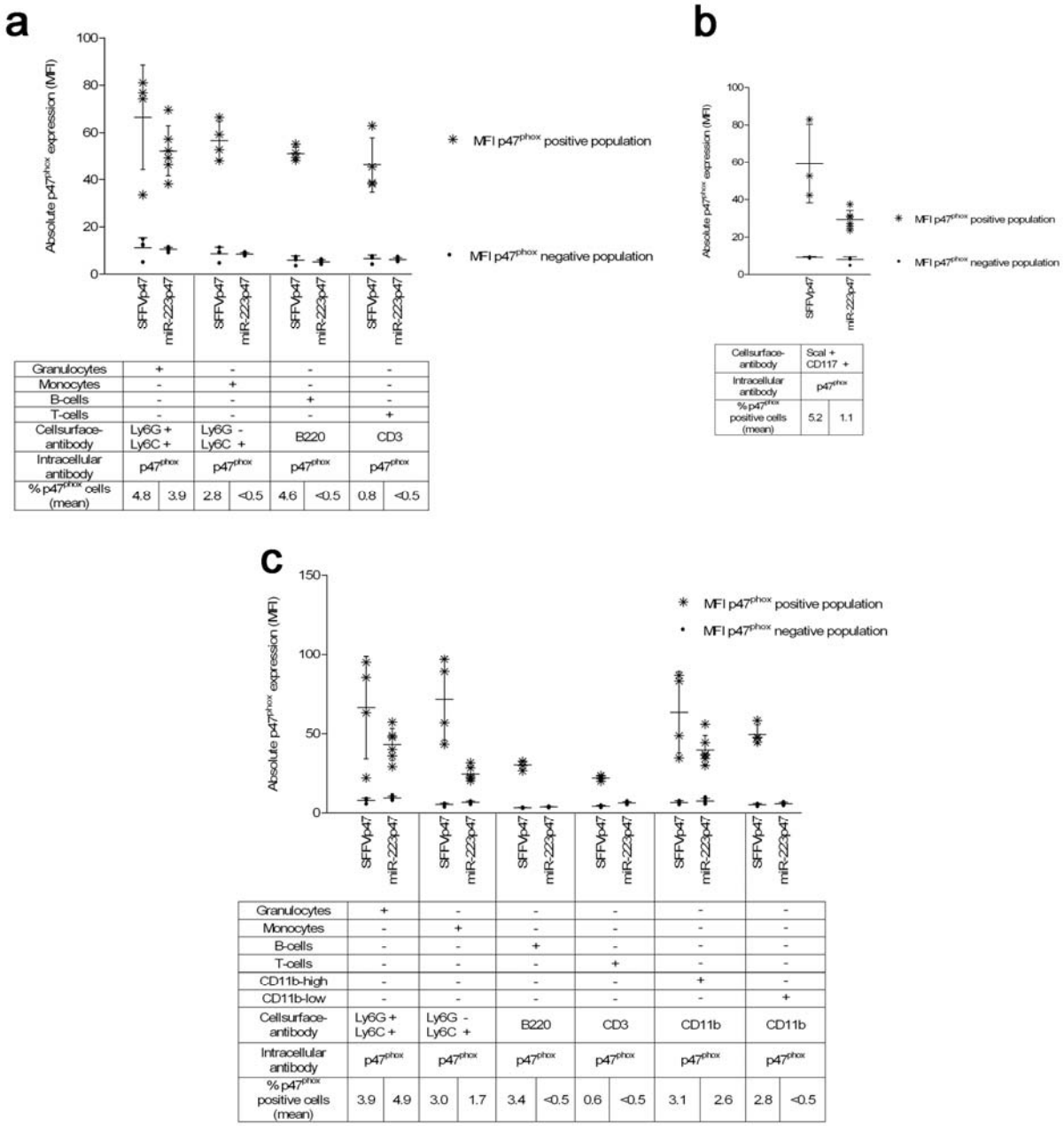

Figure 3 Absolute MFI of p47^{phox} transgene expression in p47^{phox} -/- mice after retroviral GT. Lin- BM cells of p47-/- mice were *ex vivo* γ-retrovirally transduced and transplanted in lethally irradiated p47^{phox} -/- mice. 6 weeks after transplantation p47^{phox} transgene expression was monitored in individual hematopoietic cell populations of blood, spleen and BM by FACS analysis.
(**a**) Absolute p47^{phox} expression (FACS MFI) in individual hematopoietic cell populations isolated from blood.
(**b**) Absolute p47^{phox} expression (FACS MFI) in Scal/CD117+ BM cells.
(**c**) Absolute p47^{phox} expression (FACS MFI) in individual hematopoietic cell populations isolated from spleen.
MFI of p47^{phox} positive and negative populations of each individual mouse was analyzed. Mean of percentage (%) of p47^{phox} positive cells are indicated. Bars represent mean MFI values for each group. Values below 0.5% were beyond detection limit for quantitative MFI analysis.

Functional reconstitution of NADPH oxidase activity after GT in p47^{phox} -/- mice

The production of reactive oxygen species (ROS) in granulocytes can be monitored by the dihydrorhodamine-123 (DHR) assay. In this assay, the oxidation of non-fluorescent DHR 123 to fluorescent rhodamine by ROS allows the detection of ROS producing cells by FACS analysis. Therefore we applied this methodology to analyze whether performed GT is able to restore the NADPH oxidase activity in granulocytes from treated p47^{phox} -/- mice.

Upon phorbol 12-myristate 13-acetate (PMA) stimulation, the shift in DHR signal (figure 4a) demonstrated that GT with the SFFV construct or the miRNA-223 construct resulted in the restoration of the NADPH oxidase activity. Analysis of the MFI within the DHR+ cell population showed even higher MFI for granulocytes from miRNA-223 and SFFV treated mice. The percentage of DHR+ cells reflects the expected transduction rate after transduction with MOI=1.

Taken together, GT with myelospecific miRNA-223 γ-retroviral SIN vector resulted in clear myelospecific p47^{phox} transgene expression compared to the constitutive SFFV control vector. Furthermore, the miRNA-223 γ-retroviral SIN vector could restore the NADPH oxidase equally effective as the SFFV control.

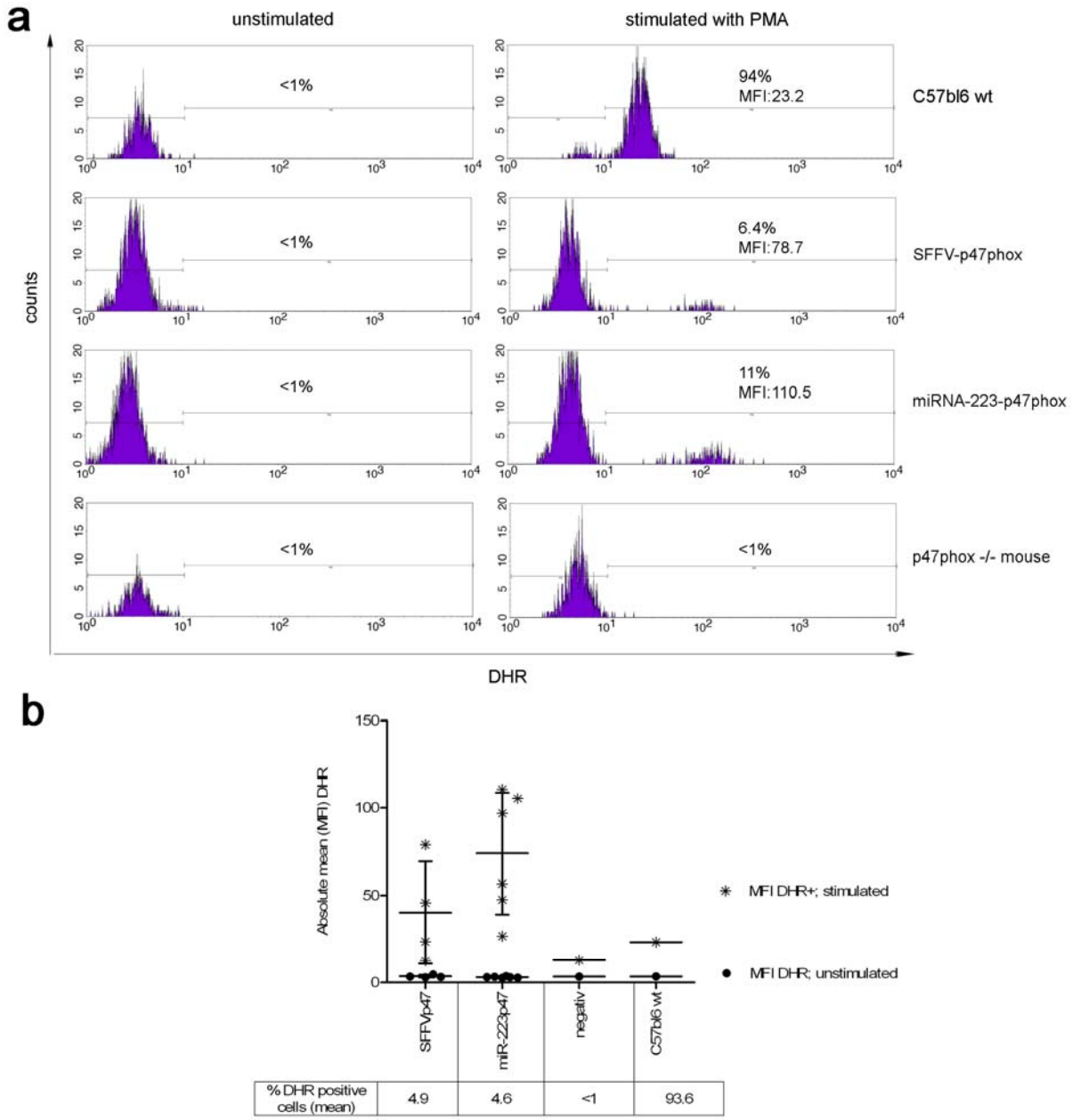

Figure 4 Reconstitution of the NADPH oxidase activity after GT. 6 weeks after transplantation with indicated vectors murine blood from each mouse was collected. The reconstitution of the NADPH oxidase activity upon PMA stimulation was analyzed by a DHR 123 oxidation assay within the Ly6G (GR1+) cell population.
(**a**) Representative examples of FACS based DHR 123 oxidation analysis in Gr1+ cells. % and MFI of the DHR positive population within Ly6G (Gr1)+ murine neutrophils are indicated. C57bl6 wt are healthy control mice.
(**b**) MFI of the DHR+ population in stimulated and unstimulated granulocytes. MFI of each analyzed mouse is shown. Bars represent mean values for each group. Mean of the percentage (%) of DHR+ cells within Ly6G (Gr1)+ murine neutrophils are indicated.

Discussion

We successfully conducted a GT in p47^{phox} -/- CGD mice using a myelospecific γ-retroviral SIN vector. In this vector, the transgene p47^{phox} expression was under control of the myelospecific miRNA-223 promoter. In these mice a clear myelospecific transgene expression was observed. The rationale of this approach is based on the fact that observed clonal dominance and malignant transformation resulted from the transactivation of oncogenes adjacent to the viral integration site in stem and progenitor cells. Therefore, restricting the expression of therapeutic transgene to short-lived neutrophils will decrease the likelihood of side effects like malignant transformation. This view is supported by recent studies which determined the insertional genotoxicity in cell-culture systems[14, 15]. The insertional transforming capacity was significantly reduced for SIN vectors compared to corresponding LTR-driven vectors. In addition, the use of cellular promoters instead of retroviral enhancer-promoters in SIN vectors lowered the risk of genotoxic side effects.

We previously analyzed the p47^{phox} expression by our vector constructs *ex vivo* in stem cells and in granulocytes (chapter 1). The miRNA-223 promoter construct showed a significant upregulation upon granulocytic propagation *ex vivo*. The same was true in this *in vivo* study. A higher p47^{phox} MFI could be observed in granulocytes compared to stem-and progenitor cells. Very low or no transgene expression could be observed in monocytes, B-lymphocytes and T-lymphocytes. These observations are in line with the description of miRNA-223 transcription in wild type mice. Transcription of mature miRNA-223 was detected at low levels in pluripotent stem- and progenitor cells and monocytes, at high levels in peripheral blood granulocytes and not detected in B and T lymphocytes[16]. CD11b antigen has been identified as myelospecific differentiation marker. It is expressed selectively on the surface of neutrophils, mature monocytes, macrophages and natural killer cells[17]. In our hands, a high CD11b cell surface expression correlated with a high MFI p47^{phox} transgene expression in the miRNA-223 group. This provides further evidence for myelospecific transgene expression.

In our study, we have shown that the p47^{phox} transgene is expressed and that it is functional. Isolated granulocytes from treated p47^{phox} -/- mice showed a reconstitution of the NADPH oxidase function. In the DHR test the shift in the FACS signal for the miRNA-223 group was comparable to the shift observed for the strong and constitutively active SFFV promoter. As the p47^{phox} subunit is involved in the regulation of the NADPH oxidase activity, one might hypothesize whether the p47^{phox} subunit represents the limiting factor for NADPH oxidase. Artificial overexpression might therefore result in the exceeding of the natural NADPH oxidase activity.

Already 5% of functional granulocytes are sufficient to protect CGD patient against infections[18-20]. Therefore GT, with the myelospecific miRNA-223 SIN vectors as it was used

in this study could lead to a sufficient number of corrected cells with sufficient NADPH oxidase activity. The presented combination of a γ-retroviral SIN vector with the myelospecific miRNA-223 internal promoter represents a major safety improvement as it minimizes the risk of malignant transformations of stem and progenitor cells as longterm effect of insertional mutagenesis.

Materials and methods

Cell culture

Non-adherent growing mouse pro B cell line BA/F3 (ACC 300) cells were grown in RPMI 1640 (PAA Laborotories), 2% Antibiotic/Antimycotic (PAA Laborotories) supplemented with 10ng/ml murine interleukin (IL) 3 (Peprotech).

Murine $p47^{phox}$ -/- Lin- BM cells were harvested from femora and tibiae of $p47^{phox}$ -/- mice (B6(Cg)-Ncf1m1J/J) (Jackson Laboratory) and enriched by the "Lineage Cell Depletion Kit" (Miltenyi Biotec). Isolated BM cells were cultured in StemSpan H3000 medium (StemCell Technologies) supplemented with 50 ng/ml murine stem cell factor (mSCF) (Peprotech), 100 ng/ml human Flt-3 ligand (Peprotech), 100 ng/ml human IL-11 (Peprotech), 20 ng/ml murine IL-3 (Peprotech), 1% penicillin/streptomycin (GIBCO), 2 mM glutamine (GIBCO)[21] and 2% FBS (Hyclone Thermoscientific).

Cloning of γ-retroviral vectors and production of infectious particles

The γ-retroviral pSERS11M8delN91s SIN vector (a derivate of the SERS11.SF.GFP.W[11], kindly provided by M. Grez, Georg Speyer, Frankfurt) was used as basic construct. pSERS11M8delN91s SIN vector contains the internal mrp8 promoter 5' to the $gp91^{phox}$ coding reading frame (CRF). In the first step a Sal I-restriction site was inserted between the $gp91^{phox}$ CRF and the Woodchuck hepatitis virus post-transcriptional element (WPRE) by *in vitro* mutagenesis (Stratagene). Then, the $gp91^{phox}$ CRF was substituted by human $p47^{phox}$ CRF (M25665, codon optimized by GENEART) by BamH1/SalI (New England Biolabs (NEB)) restriction digestion. The miRNA-223 promoter was amplified from the plasmid "223-1 gps W" (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany) and cloned into this vector by replacing the NdeI/BamHI mrp8 promoter fragment. The constitutive active promoter sequence SFFV was amplified from SERS11.SF.GFP.W (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany).

For construction, the recombinant plasmids were introduced by electroporation (Gene Pulser, Biorad) or heat shock into E. Coli Top-10 competent cells (Invitrogen). DNA manipulations were performed according to standard procedures by using commercial kits (Qiagen, Macherey-Nagel, Genomed). All resulting constructs were controled by restriction digestion followed by agarose gel electrophoresis. The cloning sites and flanking sequences of the resulting constructs were confirmed by DNA sequencing (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems (AB)).

Ecotropic infectious particles were generated by transient transfection of Phoenix (ΦE) cells (ACC 3444). These cells are derived from human embryonic kidney (HEK) 293T cells by stable integration of a plasmid carrying the moloney murine like virus (MLV) *gag-pol* CRFs and with an ecotropic MLV *env* expressing plasmid. In a 10 cm petridish, 70-80% confluent Phoenix E cell layers were co-transfected by 4μl TransIT-293 reagent (Mirus LCC Bio) per μg (total DNA), 10μg pUMVC (encoding γ-retroviral GagPol), 4μg M187 (encoding ecotropic envelope) and 40μg gene transfer vector. Infectious particle containing cell culture supernatants were collected 24h, 48h, 60h after transfection and sterile filtered (Millex Syringe Driven Filter units, 0.45 μm pore size (Millipore)). The supernatant was concentrated (30X) (Amicon-Ultra 15 centrifugal units, 1500g, 18min at RT) from 15ml to an end volume of 500μl and frozen immediately in form of aliquots a 20μl at -80°C. For titration, serial dilutions of viral supernatants were used to infect mouse pro B cells BA/F3 (ACC 300) in the presence of protamine sulfate (8μg/ml, Sigma). Virus supernatant was thawed and taken immediately for transduction (spinoculation for 90 min, 2500 RPM, at 32°C). One week after transduction DNA was extracted from the cell pellets by „DNeasy blood and tissue kit" (Qiagen). The vector copy number was quantified by real-time q-PCR with 50-200 ng template DNA in a reaction volume of 25μl. The set of primers and probes used for the analysis were as follows (table 2):

detect	primers/probes	sequence	final concentration, nM	company
actb (encoding β-actin) (endogenous control)	forward primer	5'-AGAGGGAAATCGTGCGTGAC-3'	500	Microsynth
	reverse primer	5'-CAATAGTGATGACCTGGCCGT-3'	500	Microsynth
	probe	VIC-CACTGCCGCATCCTCTTCCTCCC-MGB	200	AB
WPRE	forward primer	5'-TTTCTGGGACTTTCGCTTTCC-3'	500	Microsynth
	reverse primer	5'-AGGCGGCGATGAGTTCTG-3'	500	Microsynth
	probe	FAM-CCTCCCGATCGCC-MGB	200	AB

Table 1 Primers and probes used for real-time q-PCR.

We generated a standard curve by combining DNA from BA/F3 cells without and with one integration per cell (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany). TaqMan reactions were carried out in triplicates in an ABI Prism 7700 HT Sequence Detection System (AB). Transduction of HSC and different cell lines (e.g. BA/F3 cells) with one and the same virus supernatant resulted in different transduction efficiencies. Therefore we determined the factor between the titers related to BA/F3 cells and to HSC. Murine Lin- BM cells and BA/F3 cells were transduced with the SFFV control virus supernatant and analyzed 5 days later by intracellular $p47^{phox}$ staining

measured by FACS (BD). The titer in BA/F3 cells was calculated to be 1.6 fold higher compared to HSCs.

Detection of transgene expression in individual hematopoietic cell populations

$p47^{phox}$ expression was measured by performing ICS with the anti-$p47^{phox}$ antibody (clone 1), APC-labeled by BD-services. For combined staining of surface markers and intracellular $p47^{phox}$, the cells were first blocked with mouse Fc-block (BD) (clone 2.462) for 10min at 4°C, incubated with the surface antibodies (see table 2) for 1h at 37°C and washed twice. Thereafter, $p47^{phox}$ was stained with APC anti-$p47^{phox}$ using the Cytofix/Cytoperm Kit (BD) according to manufacturer's instructions. APC mIgG1 Isotyp (clone X40, BD) antibody was utilized as isotype control in intracellular staining.

Detection of murine	antibody	clone	company
granulocytes	FITC anti-Ly6G	1A8	BD
	PE anti-Ly6G and Ly6C	RB6-8C5	BD
monocytes	FITC anti-Ly6G	1A8	BD
	PE anti-Ly6G and Ly6C	RB6-8C5	BD
B-lymphocytes	FITC anti-CD45R/B220	RA3-6B2	BD
T-lymphocytes	PE anti-CD3	17A2	BD
neutrophils, monocytes, natural killer cells	PE CD11b	M1/70	BD
progenitor-and stemcells	Ly-6A/E ScaI	E13-161.7	BD
	FITC CD117	2B8	ebioscience
isotype control	PE ratIgG2a, κ	R32-95	BD
	FITC ratIgG2b, κ	A95-1	BD

Table 2 Surface antibodies utilized for detection of individual murine hematopoietic cells.

Determination of γ-retroviral copy number by q-PCR in p47^{phox} -/- mice

B-lymphocytes, T-lymphocytes and monocytes from each p47^{phox} -/- mouse were sorted from 5*10^6 splenocytes by live, sterile cell sorting (FACS ARIA, BD).

Genomic DNA was extracted from the cell pellets by „Qiamp DNA Mini kit“ (Qiagen). The vector copy number was quantified by two parallel real-time q-PCR reactions in one tube with 20-50 ng template DNA in a reaction volume of 20μl. The set of primers and probes used for the analysis were as follows (table 3):

detect	primers/probes	sequence	final concentration, nM	company
actb (encoding β-actin) (endogenous control)	forward primer	5'-ATTGCCACCACCTGTCAACT-3'	300	Operon
	reverse primer	5'-GCAACCTAGCCCCTGTCC-3'	750	Operon
	probe	RED-CACTGCCGCATCCTCTTCCTCCC-BHQ2	80	Operon
WPRE	forward primer	5'-ATTGCCACCACCTGTCAACT-3'	200	Operon
	reverse primer	5'-GCAACCTAGCCCCTGTCC-3'	200	Operon
	probe	RED-CACTGCCGCATCCTCTTCCTCCC-BHQ2	50	Operon

Table 3 Primers and probes used for real-time q-PCR.

We generated a standard curve by combining DNA from BA/F3 cells without and with one integration per cell (kindly provided from M. Grez, Georg-Speyer-Haus, Institute for biomedical research, Frankfurt, Germany). TaqMan reactions were carried out in duplicates in a LighCyler 480 II (Roche).

Transduction of murine p47^{phox} -/- Lin- BM cells

p47^{phox} -/- Lin- BM cells were isolated using the lineage cell depletion kit (Miltenyi). The BM cells were cultured for 2 to 3 days in StemSpan H3000 supplemented with cytokines (see cell culture section). Cell culture plates were precoated with Retronectin (TaKaRa) by incubation with 10 μg/cm^2 at RT for 2h followed by HSA incubation for 10 min. Frozen titered γ-retroviral supernatants were thawed, prediluted in StemSpan H3000 and loaded onto the precoated plates by centrifugation (3000 RPM for 30 min at 4°C). Then, p47^{phox} -/- Lin- BM cells were seeded onto virus-loaded plates and cultured for 2 to 3 days in cytokine supplemented StemSpan H3000[21].

Transplantation of transduced Lin- p47^{phox} -/- BM cells into p47^{phox} -/- mice

4-6 weeks old p47^{phox} -/- mice were lethally irradiated with 950cGy. Per mouse 1E6 γ-retrovirally transduced Lin- BM cells were injected 4 hours later via tail vein injection. All mice received neomycin (SIGMA) treatment as additive in the drinking water (1.67 mg/ml) for six weeks. After six weeks, murine blood from the tail vein was analyzed for respiratory burst activity (see analysis of respiratory burst activity). Then, mice were euthanized with CO_2. Blood, spleen, BM from each mouse were taken immediately and analyzed for p47^{phox} expression by FACS. Prior to surface and intracelluar FACS staining, erythrocytes in blood were lysed by ammonium chloride lysing buffer (Kantonsapotheke Zürich). Splenocytes were harvested by passing through a 70μM cell strainer (BD) followed by ammonium chloride treatment. BM cells were flushed out from femora and tibiae followed by erythrocyte lysis. All mice were kept at the Institut für Labortierkunde of University hospital Zurich under specific pathogen-free conditions. All animal procedures were conducted in accordance with the ethical guidelines of Animal Studies Ethics Committee and in concordance with Swiss animal protection law.

Analysis of respiratory burst activity of neutrophils from murine blood samples

For analyses of respiratory burst activity, the dihydrorhodamine assay (DHR) 123 was carried out as described[8] with a few modifications. We combined the FACS based DHR assay with the analysis of cell surface markers. 100-200μl mouse blood were collected by tail venisection. Murine neutrophils were identified by prestaining with PerCP-Cy5.5 anti-Ly-6G and Ly-6C (clone RB6-8C5, BD) for 10min at 4°C. Then erythrocytes in blood were lysed with pre-cooled ammonium chloride lysis buffer for 10 min on ice. Afterwards, the cells were washed and incubated in 500μl Hanks' Buffered Saline Solution (HBSS) (PAA Laboratories) with Ca and Mg supplemented with 0.5% HSA, CSL Behring), 7.5 mM Glucose (SIGMA), 2μl catalase (1000U/ml, SIGMA), 10 μl DHR (stock 29mM, 1:1000 diluted, SIGMA) for 15min at 37°C. Blood cells were stimulated with 1μg/ml PMA (SIGMA) for 15min at 37°C in presence of DHR. A shift in DHR 123 fluorescence was measured by flow cytometry (BD) within 30 min after stimulation.

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Chapter 3:
Signed outside:
A surface marker system for transgenic cytoplasmic proteins

submitted to Journal of Molecular Therapy

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Contributions: All experiments were conducted by Vital Wohlgensinger in Zürich. Vital Wohlgensinger and Ulrich Siler analyzed the results. All figures in this chapter were designed by Vital Wohlgensinger and Ulrich Siler. Ulrich Siler designed the research.

Abstract

Chronic Granulomatous Disease (CGD) comprises a group of five primary immunodeficiencies characterized by an impaired respiratory burst activity of myeloid cells. We are currently developing a gene therapy (GT) vector for the $p47^{phox}$-deficient form of CGD. Classic intracellular immunostaining (ICS) of the cytoplasmic $p47^{phox}$ transgene product, however, interferes with respiratory burst activity. Here we report a new system for measuring $p47^{phox}$ expression: A single open reading frame (ORF) encoding the surface marker protein ΔLNGFR (truncated low-affinity nerve growth factor receptor) linked to the $p47^{phox}$ transgene *via* the 2A oligopeptide co-expression technology. Translation generates two discrete products: $p47^{phox}$ localizing to the cytoplasm and [ΔLNGFR-2A] localizing to the cell surface. Six weeks after transplantation of transduced autologous hematopoietic stem cells (HSC) into $p47^{phox}$ -/- mice, the intracellular $p47^{phox}$ FACS signal intensities corresponded with surface ΔLNGFR staining in monocytes, B cells, T cells and ScaI+ bone marrow (BM) cells *in vivo*. The $p47^{phox}$ cleavage product restored NADPH-oxidase activity in granulocytes differentiated from transduced $p47^{phox}$ -/- murine hematopoietic stem cells (HSC) *ex vivo*, in murine granulocytes/monocytes *in vivo,* and in transduced human monocyte derived macrophages from $p47^{phox}$-deficient CGD patients. In conclusion this new marker system allows highly efficient, indirect detection of cytoplasmic transgene products by FACS surface staining.

Introduction

CGD results from impaired respiratory burst activity caused by a mutated phagocyte NADPH oxidase complex [1]. This enzyme complex consists of two membrane spanning subunits ($gp91^{phox}$ and $p22^{phox}$) plus three cytosolic components ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$). Approximately 60% of all CGD cases have been estimated to result from mutations in the gene encoding $gp91^{phox}$ with an additional 30% caused by gene mutations encoding $p47^{phox}$ [2].

The $p47^{phox}$ subunit is a cytoplasmic protein recruited to the transmembrane NADPH-oxidase complex upon respiratory burst induction. Currently, a GT vector for the $p47^{phox}$-deficient form of CGD is being developed. Critically, the efficacy of GT should be monitored *via* the detection of transgenic $p47^{phox}$ expression.

Since cellular integrity is an absolute pre-requisite for the induction of phagocytic NADPH-oxidase activity, it is impossible to combine functional analyses with the detection of $p47^{phox}$ protein by immunostaining. To overcome this limitation we developed a system in which the expression of the cytoplasmic transgene product is linked to, and can be monitored by, the coexpression of a cellular surface marker. A single ORF was created encoding the

therapeutic $p47^{phox}$ transgene linked to the ΔLNGFR surface marker protein via the Foot and Mouth Disease Virus (FMDV) 2A peptide. In this 2A-mediated co-expression system the detection of the surface marker provides a indirect measure for the expression of the cytoplasmic transgene product.

Results

The ΔLNGFR surface marker and the therapeutic p47^{phox} transgene product are synthesized in a 1 : 1 ratio

The p47^{phox} marker construct was assembled to encode a single, self-processing, polyprotein comprising three domains; (i) an N-terminal domain of ΔLNGFR (NM_002507; nts. 114-944), (ii) the 2A oligopeptide (31aa; X00871; nts. 3483-3575) and (iii) the C-terminal domain comprising the p47^{phox} subunit of the NADPH-oxidase (M25665, codon optimized) (figure 1a). Cotranslational ribosomal 'skipping' mediated by 2A is predicted to produce two translation products: the transmembrane ΔLNGFR fused to the 2A peptide ([ΔLNGFR-2A]), and the cytoplasmic p47^{phox} protein with an N-terminal proline (P-p47^{phox}).

To test this marker system and its influence on the transgenic protein activity, murine 32D hematopoietic progenitor cells (German Collection of Microorganisms and Cell Cultures (DSMZ), DSMZ no.: ACC 411) were transduced using a recombinant gammaretrovirus encoding the marker construct. Transgene expression was confirmed by FACS analysis. Surface staining of [ΔLNGFR-2A] and staining of intracellular p47^{phox} demonstrated a transduction rate of 22% in each case. As expected, surface [ΔLNGFR-2A] staining plus intracellular p47^{phox} double-staining revealed exclusively [ΔLNGFR-2A]/P-p47^{phox} double-positive and double-negative cells, whereas P-p47^{phox} could not be detected by surface staining (figure 1b).

To confirm co-translational 'cleavage', protein extracts of ΔLNGFR-2A-p47^{phox} transduced 32D cells were analyzed by Western blotting. P-p47^{phox} linked to [ΔLNGFR-2A] showed a very weak band with identical molecular weight in both blots, corresponding to the expected size of the uncleaved polyprotein (figure 1c, filled triangles). Strong bands were detected for both cleavage products [ΔLNGFR-2A] and P-p47^{phox} (figure 1c, open triangles), with masses of ~65kDa and ~47kDa, corresponding to the control extracts. Signal intensities of both Western blots indicate that >98% of the transgene encoded proteins were synthesized as separated (cleaved) proteins due to ribosome 'skipping' mediated by the 2A sequence.

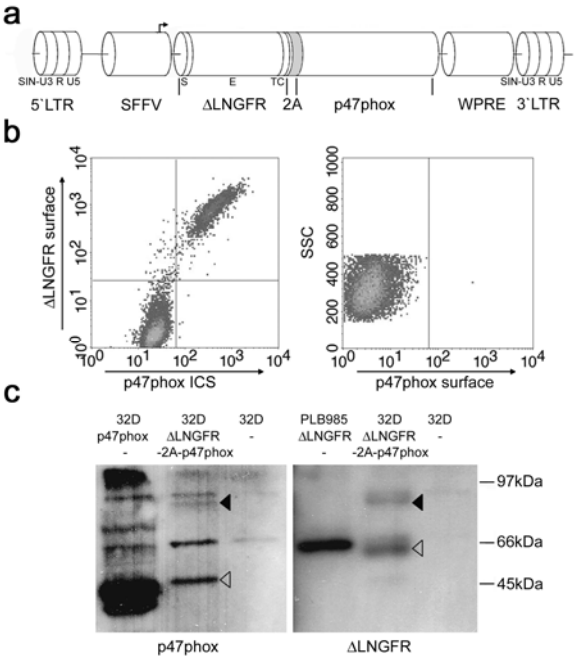

Figure 1 Cloning strategy and detection of the two translation products of the marker system.
(a) Integrated gammaretroviral SIN-vector with constitutive spleen focus-forming virus (SFFV) promoter driving ΔLNGFR-2A-p47^{phox} expression (S: LNGFR signal peptide, E: LNGFR extracellular domain, T: LNGFR transmembrane domain, C: shortened LNGFR cytoplasmic domain, 2A: FMDV 2A peptide (31aa), WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element).
(b) FACS analysis of transduced 32D progenitor cells (ICS: intracellular staining).
(c) Western blot analysis of transduced 32D cells detecting p47^{phox} and LNGFR. 32D cells transduced with p47^{phox} and a monoclonal clone derived from transduced PLB985 cells expressing ΔLNGFR were used as positive controls. Filled triangle: ΔLNGFR-2A-p47^{phox} fusion protein, open triangle: ΔLNGFR fused to 30aa 2A peptide ([ΔLNGFR2A]), and the cytoplasmic p47^{phox} protein with an N-terminal proline (P-p47^{phox}) translation products.

The ΔLNGFR surface epitope correlates with the presence of the cytoplasmatic p47^{phox} protein in vivo

Lineage-negative (Lin-) BM cells from p47^{phox} -/- mice were isolated, retrovirally transduced and reinfused into lethally irradiated p47^{phox} -/- mice. The transduction rate was 4.2%, as determined by p47^{phox} ICS of Ly-6G(1A8)-positive granulocytes obtained after *ex vivo* differentiation of transduced BM cells in liquid cell culture. Six weeks after reinfusion into p47^{phox} -/- mice, p47^{phox} and ΔLNGFR expression was analyzed. As expected, random integration into the genome resulted in heterogeneous expression levels, also known as 'position-effect variegation' [3].

In blood the mixed granulocytic/monocytic population of Ly-6G and/or Ly-6C-positive cells recognized by anti-Ly-6G/Ly-6C mAb RB6-8C5 revealed populations with varying p47^{phox} and ΔLNGFR stain intensities (figure 2a). In the spleen, granulocytes and monocytes were identified separately by anti-Ly-6G/Ly-6C (mAb RB6-8C5) and anti-Ly-6G (mAB 1A8) double staining. Granulocytes (RB6-8C5+/1A8+) varied in ΔLNGFR staining intensity (figure 2b). In splenic monocytes (RB6-8C5+/1A8-), however, the intracellular p47^{phox} signal ran in parallel to the surface ΔLNGFR staining (figure 2c). The same parallel between the p47^{phox} and ΔLNGFR signals were observed within a hematopoietic stem- and progenitor cell enriched population of Scal+ BM cells (figure 2d), in splenic B220+ B-cells (figure 2e) and in CD3+ T-cells (figure 2f). Taken together, with the exception of granulocytes, the intracellular p47^{phox}

FACS signal ran in parallel to the ΔLNGFR surface marking in non ΔLNGFR/p47^{phox} double-negative cells.

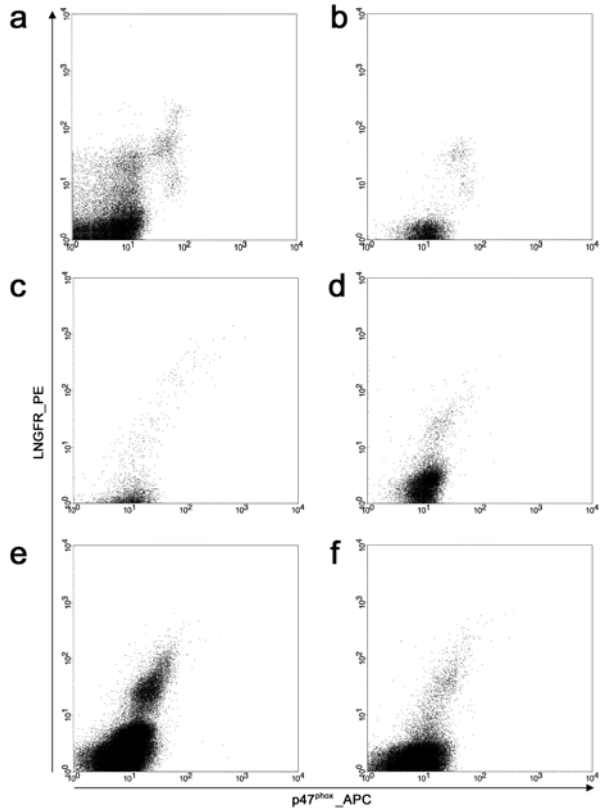

Figure 2 FACS analysis of murine blood, spleen and BM cell populations six weeks after GT
Surface ΔLNGFR / intracellular p47^{phox} double-staining of **(a)** mouse blood gated for Ly-6G and/or Ly-6C-positive cells (mAb clone RB6-8C5) i.e. mainly granulocytes and monocytes, of **(b)** splenic granulocytes (Ly-6G/Ly-6C (mAb RB6-8C5) / Ly-6G (mAb 1A8) double positive cells), of **(c)** splenic monocytes (Ly-6G/Ly-6C (mAb RB6-8C5) positive / Ly-6G (mAB 1A8) negative cells), of **(d)** hematopoietic stem- and progenitor cell enriched population of ScaI+ BM cells, of **(e)** splenic B220+ B-cells and of **(f)** splenic CD3+ T-cells.

The P-p47^{phox} translational-skipping product restores respiratory burst activity *in vitro* and *in vivo*

We next investigated whether the P-p47^{phox} translational skipping product was able to restore respiratory burst activity. The ΔLNGFR-2A-p47^{phox} retrovirus vector was used to transduce Lin- BM cells obtained from p47^{phox}-/- mice and subsequently differentiated to granulocytes *ex vivo*. Granulocytes derived from untransduced and transduced p47^{phox}-/- Lin- BM cells were stimulated with PMA and the NADPH-oxidase activities visualized by a nitroblue tetrazolium (NBT) assay. Within the transduced cell population the reduction of NBT to its formazan derivative in individual cells yielded intracellular blue/black precipitates demonstrating restoration of the NADPH-oxidase activity (figure 3b) - not observed in the negative-control (figure 3a).

Six weeks after reinfusion of transduced Lin- BM cells into p47^{phox} -/- mice, blood samples were analyzed for respiratory burst activity. Mouse blood was stained with the Ly-6G/Ly-6C specific mAb clone RB6-8C5. Ly-6G/Ly-6C-positive cells were analyzed for ΔLNGFR expression and their respiratory burst activity upon PMA stimulation was determined by the

FACS based dihydrorhodamin-123 (DHR) oxidation assay. As expected, unstimulated Ly-6G/Ly-6C+ blood cells were partially ΔLNGFR-positive and DHR-negative (figure 3c). Upon stimulation, the shift in DHR signal demonstrates activation of the NADPH oxidase and reconstitution of respiratory burst activity in neutrophils and monocytes derived from transduced stem cells *in vivo* (figure 3d). Those Ly-6G/ΔLNGFR-positive cells which do not show a shift in DHR signal are expected not to represent monocytes or granulocytes as the anti- Ly-6G/Ly-6C mAb clone RB6-8C5 also detects Ly-6C-positive dendritic cells and subpopulations of lymphocytes[4] with no respiratory burst activity.

Recently it has been reported that gammaretroviral transduction of macrophages is possible, although with low efficiency[5]. Therefore we analyzed the activity of the construct in human macrophages derived from a p47^{phox}-CGD patient (figure 3e,f,g). Monocytes were isolated and differentiated into macrophages for seven days. Macrophages were then transduced, and analyzed for NADPH-oxidase activity three days later. As expected[5], transduction efficiency was low. As in the murine model, we detected NBT-positive cells in transduced human cells. This clearly indicates that the cytoplasmic P-p47^{phox} translational skipping product is able to restore the NADPH-oxidase activity not only in primary murine cells (both *ex vivo* and *in vivo*) but also in derived primary human cells.

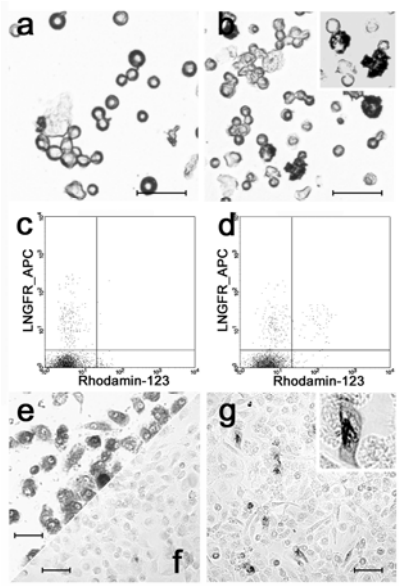

Figure 3 Functional reconstitution of respiratory burst activity by the P-p47^{phox} translation product.
Light microscopic evaluation of granulocyte nitroblue tetrazolium (NBT) assay prepared by cytospin. Intracellular dark precipitates indicate respiratory burst activity. Granulocytes derived from untransduced **(a)** and ΔLNGFR-2A-p47^{phox} transduced **(b)** Lin- BM cells from p47^{phox} -/- mice after differentiation in liquid cell culture (Scale bars = 50μm).
(c, d) Combined staining of Ly-6G/Ly-6C+ granulocytes and monocytes in mouse blood and functional analysis of respiratory burst activity by dihydrorhodamin-123 (DHR) assay. Please note that Ly-6C recognized by mAb clone RB6-8C5 is also present on dendritic cells and subpopulations of lymphocytes[4] without respiratory burst activity. **(c)** DHR test of unstimulated and **(d)** PMA stimulated Ly-6G/Ly-6C+ cells in mouse blood six weeks after ΔLNGFR-2A-p47^{phox} GT.
NBT assay on macrophages from healthy control **(e)**, or from untransduced **(f)** and ΔLNGFR-2A-p47^{phox} transduced **(g)** macrophages derived from a p47^{phox}-deficient CGD patient (Scale bars = 100μm). Inserts in **(b)** and **(g)** show NBT-positive cells in higher magnification.

Discussion

The 2A co-expression methodology was applied to overcome the limitation that detection of the cytoplasmic p47^{phox} transgene product interferes with functional analysis. Our expression strategy expands the panel of intracellular marker proteins from co-expressed GFP[6] to surface epitopes in general. A perfect marker molecule must consist of a transmembrane domain plus an extracellular epitope. Such a marker should be; (i) biologically inert, (ii) not participate in signal transduction and (iii) not dimerize with transmembrane receptors (and thereby potentially contribute to signal transduction processes). Finally, in order to avoid immunogenicity after human GT the marker molecule should be of human origin and normally expressed in a tissue different from the GT target tissue. In the present vector the expression of the ΔLNGFR surface marker was linked with the expression of a cytoplasmic p47^{phox} transgene.

Double labeling of intracellular p47^{phox} and surface standing ΔLNGFR in 32D hematopoietic progenitor cells revealed their co-expression in cells transduced with the retroviral vector. The signal intensities of both Western blots indicate that >98% of the transgene encoded proteins were synthesized as separated (cleaved) proteins due to ribosome 'skipping' mediated by the 2A sequence. Just 16-20aa of FMDV 2A possess skipping activity[7], although N-terminal extensions increase the activity up to >99% for 2A sequences of 33aa or longer[6, 8, 9]. The cleavage efficiency of the present construct containing 31aa of 2A is consistent with efficiencies previously reported. Due to the high cleavage efficiency of our marker system the detection of the [ΔLNGFR-2A] surface marker provides a direct measure for the presence of the cytoplasmic p47^{phox} transgene product. Moreover, the cleavage process of a translation product encoded in a single open reading frame dictates a 1:1 ratio of the two cleavage products. Our mouse experiments showed position-effect variegation ran in parallel for the therapeutic protein product (intracellular p47^{phox}) and the cell surface marker (ΔLNGFR) in hematopoietic stem/progenitor cells, monocytes, T cells and B cells. Surprisingly, the intracellular p47^{phox} / surface ΔLNGFR double staining revealed individual populations in granulocytes differing in ΔLNGFR staining intensities. The explanation for this observation is unclear and a detailed analysis ongoing.

The use of intracellular ribosomal entry sites (IRES) represents an alternative approach to generate multicistronic expression from one vector. In a bicistronic IRES-based vector, translation initiation of the 5' coding reading frame (CRF) requires the m^{7}G cap and its associated protein factors, whereas the 3' CRF is thought to be translated cap-independently by direct recruitment of the ribosome to the IRES element. Due to different efficiencies of translation initiation the ORF downstream of IRES is consistently less expressed than the ORF upstream of IRES [10]. Compared to IRES based biscistronic expression, GFP co-expression by the 2A strategy was approximately four times higher[11].

Additionally, the packaging capacity is limited in retroviral vectors[12]. Regarding size limitations the 19AA/57nt 2A sequence is advantageous compared to e.g. 470nt IRES of FMDV or the 640nt IRES of Poliovirus. Furthermore, both IRES sequences were reported to result in retroviral titer reduction whereas 2A did not[13]. One reason for this reduction in retroviral titers by IRES sequences might be their secondary structure. The hepatitis C virus-like picornavirus IRES elements are known to possess extensive RNA secondary structure identified as substrate for the Dicer ribonuclease[14]. A ribonucleolytic cleavage by Dicer would interfere with the retroviral life cycle.

In our vector, ΔLNGFR serves as a membrane standing marker epitope indirectly representing the cytoplasmic P-p47^{phox} translation product. The ΔLNGFR marker protein was used previously until Li and coworkers reported one leukemic transformation event in a single mouse by a ΔLNGFR containing gammaretroviral LTR-driven (non-SIN) vector after integration into the murine *evi1* gene associated with Evi1 expression[15]. However, data amassed from 17 independent investigators from >300 mice having received BM transplantation of HSCs transduced with ΔLNGFR expressing retroviral vectors revealed no adverse events. This weakens the argument for an increased frequency of insertional oncogenesis by ΔLNGFR[16]. Malignant transformation is a multi-step process[17], in which retroviral insertion may be just one event[18]. Evi1 over-expression resulting from retroviral insertion near *evi1* has been reported to interact with mutant *aml1* to induce MDS/AML[19]. Interestingly, ΔLNGFR had been used in two clinical trials as marker molecule for the gammaretroviral transduction of patient T-cells (mentioned in[20]). Cumulatively, the 46 patients treated since 1994 received a total of >10^{11} genetically modified T-cells. Expansion (up to 40% of circulating cells) and long-term persistence (>10 years) of transduced T-cells were observed in these patients in the absence of any adverse event or toxic effect related to the gene transfer procedure. A detailed follow up after 10 years of two patients revealed that despite the observed frequency of insertional gene activation, there was no evidence of clonal selection or preferential survival of transduced T cells[20]. These facts lead to the conclusion that the leukemia was induced in Li's mouse[15] by vector LTR-mediated Evi1 over-expression only in combination with an undetected preexisting or acquired additional hit, but not by ΔLNGFR alone. ΔLNGFR thus represents a useful marker protein not only for preclinical gene transfer studies involving therapeutic cytoplasmic proteins but might potentially also be applied in future clinical GT using retroviral SIN vectors. Nevertheless, the presented cloning strategy is not limited to ΔLNGFR. The 2A methodology can be adapted to other transmembrane marker proteins under maintenance of the 1:1 ratio of synthesized therapeutic protein and marker protein. After cotranslational ribosomal 'skipping' the cytoplasmic skipping product consisted of the therapeutic p47^{phox} transgene product N-terminally prolonged by one proline (P-p47^{phox}). We have shown by functional assays that the

biological activity of $p47^{phox}$ is not affected by the addition of an N-terminal proline residue derived from the 2A sequence combining ΔLNGFR and $p47^{phox}$.

In summary, the presented cytoplasmic/surface marker co-expression technology allows highly efficient, indirect detection of cytoplasmic transgene products by FACS surface staining. The cloning strategy can be easily adapted to different cytoplasmic therapeutic transgene products and alternative surface marker proteins, and it provides a useful tool for the detection of cytoplasmic transgene products optionally applied for GT monitoring.

Materials and Methods

Vectors

The gammaretroviral vector backbone was derived from self-inactivating (SIN) gammaretroviral SERS11.SF.gp91.W (gift M.Grez). The transgene was exchanged against a cassette containing the N-terminal domain of ΔLNGFR (NM_002507; nts. 114-944), the 2A oligopeptide (31aa; X00871; nts. 3483-3575) followed by the $p47^{phox}$ subunit of the NADHP-oxidase (M25665, codon optimized).

Virus production and transduction

For retroviral vector production PhoenixE cells or 293T cells were cotransfected with the ΔLNGFR-2A-$p47^{phox}$ gammaretroviral self-inactivating (SIN)-vector together with pUMVC plus pMD2.VSV.G or M187 (gift of M. Grez) in the presence of 10 μM chloroquin (SIGMA). Cell free virus supernatant was concentrated using Amicon-15 centrifugal filter devices with 100.000kDa cut off (Millipore).

For transduction of 32D cells the viral particles and 10^5 cells were spinoculated for 90min, 400g at 32°C with 8 μg/ml protamine sulfate (SIGMA) and incubated overnight. For transduction of mouse Lin- BM cells, the viral particles were spinoculated (400g, 30min, 4°C) onto fibronectin-coated tissue culture plates. The Lin- BM cells were prestimulated for 2 days, spun down onto the virus preloaded plates[21] and kept in culture for three days.

For transduction of human monocyte derived macrophages, monocytes were isolated from human whole blood using CD14 microbeads (Miltenyi Biotec), allowed to settle for 3h and cultured in RPMI, 10 % FBS, 1 μM GM-CSF for 7 days. Then, viral particles were spinoculated onto the adherent macrophages (90min, 400g at 32°C)[5].

Antibodies

FITC-anti-LNGFR (cloneME20.4-1.H4) and FITC anti-CD3ε (clone145-2C11) were from Miltenyi Biotec. PE anti-LNGFR (clone C40-1457), FITC anti-Ly-6G (1A8), PerCP-Cy5.5 anti-Ly-6G and Ly-6C (clone RB6-8C5), PE anti-Ly-6A/E ScaI (E13-161.7) and FITC anti-CD45R/B220 (clone RA3-6B2) were from BD Biosciences as was anti-$p47^{phox}$ antibody (clone 1) which was APC-labeled by BD-services.

Murine cells were first blocked with Fc block anti-CD16/CD32 (clone 2.4G2, BD). For combined surface/intracellular staining cells were incubated with surface antibody for 1h, washed twice, followed by intracellular staining with anti-$p47^{phox}$-APC using the

Cytofix/Cytoperm Kit (BD) according to the manufacturer's instructions. Flow cytometry analyses were performed with FACS Calibur (BD).
Western Blot analyses were performed using ECL Advanced Western Blot Detection Kits (GE Healthcare).

Mice

$p47^{phox}$ -/- mice (B6(Cg)-Ncf1m1J/J) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice were maintained under specific pathogen-free conditions. Housing and experimental protocols were in accordance with the Swiss animal protection law and in compliance with the regulations of the Veterinaeramt, Kanton Zurich. For isolation of Lin- BM cells, BM was harvested from femurs/tibias and enriched by the "Lineage Cell Depletion Kit" (Miltenyi Biotec).
For BM transplantation 4-6 weeks old mice were irradiated with 950cGy. Per mouse, 1E6 gammaretrovirally transduced Lin- BM cells were injected 4 hours later via tail vein injection. Mice received prophylactic neomycin (SIGMA) as additive in drinking water (1.67 mg/ml). For *ex vivo* granulocyte differentiation, Lin- BM cells were washed two days after transduction and cultured in RPMI, 20% FBS, 100ng/ml G-CSF and 10ng/ml IL-3 for 12 days.

Functional assays

The NBT assay and dihydrhodamine assay (DHR) 123 were carried out as described[22] with one modification: To identify murine granulocytes/monocytes in the DHR 123 assay we first stained the blood samples using mAB clone RB6-8C5 and conducted the assay with the prestained samples. FACS analysis was carried out on a FACS Calibur™ (Becton Dickinson AG, Allschwil, Switzerland) and images were captured by a Leica DM IL HC microscope (Leica Microsystems, Heerbrugg, Switzerland).

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Authorship

Contribution: V.W. performed experiments; U.S., R.S and M.D.R. designed the research; U.S., J.R. and V.W. analyzed results and made the figures; U.S., M.D.R., R.S and J.R. wrote the paper.

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General Discussion and Outlook

General Discussion

Retroviral GT provides a new approach to treat X-CGD (g91^{phox}-deficiency)[1], adenosine deaminase deficient immunodeficiency (ADA-SCID)[2] and X-linked severe combined immunodeficiency (SCID-X1)[3]. This is highly desired, as for CGD patients the present state-of-art treatment is the bone marrow transplantation (BMT). BMT has been associated with unacceptably high rates of morbidity, mortality and graft failure, except in very selected cases in which an HLA-identical donor is available[4, 5]. However, the first clinical GT studies for X-CGD and SCID were accompanied with clonal dominance[1, 6, 7] or leukemic progression[8] as long term effects of transactivation events. The observed severe side effects resulted from the transactivation of oncogenes adjacent to the viral integration site by retroviral enhancer activity in stem- and progenitor cells[8-10]. These observations demand the development of safer vector systems. For the p47^{phox}-deficent form of CGD, representing 30%[11, 12] of all CGD patients, BMT is the only available therapy at present with no GT vector being available. Therefore, in this thesis, a safer γ-retroviral GT vector for correction of p47^{phox}-CGD was developed.

To minimize the risks of transactivation we utilized γ-retroviral SIN vectors[13, 14] for GT of p47^{phox} -/- CGD (chapter 1,2). In these vectors the viral promoter/enhancer elements are inactivated upon integration. The transgene expression is driven by an internal tissue-specific promoter element which ideally lacks enhancer activity and restricts transgene expression to terminally differentiated granulocytes/monocytes. Restricting the expression of the therapeutic transgene to short-lived neutrophils will decrease the likelihood of side effects like clonal dominance or malignant transformation in stem- and progenitor cells. This view is supported by recent studies which determined insertional genotoxicity in cell-culture systems[15, 16] and *in vivo* tumor-prone mouse models[17]. The insertional transforming capacity was significantly reduced by SIN vectors compared to the corresponding LTR-driven vectors. The use of cellular promoters as internal promoter (instead of retroviral enhancer-promoters) within SIN vectors additionally lowered the risk of genotoxic side effects[16].

To develop a SIN vector for p47^{phox} CGD GT we performed two rounds of screening to find a potent internal promoter (chapter 1). In the first screen, different myelospecific promoters driving GFP expression were tested. GFP as transgene was used as there is no existent pre-myeloid p47^{phox} -/- cell line which can be propagated to granulocytes. To screen for *ex vivo* myelospecific p47^{phox} transgene expression, human BM cells (e.g. CD34+ from p47^{phox} -/- CGD patients) would have been the ideal system. However, as the p47^{phox} -/- CGD[11, 12] disease is rare, it is difficult to get enough of the desired patient cells to perform an *ex vivo* screen. Therefore we decided to use murine primary cells from p47^{phox} -/- mice for a second

screen. An earlier study has already shown that the correction of the NADPH oxidase in p47^{phox} deficient mice is possible with a LTR-driven vector carrying the human p47^{phox} cDNA[18].

At the beginning of our study the obtained SIN γ-retroviral vector titers of the myelospecific candidates, which were produced by standard calcium phosphate methodology, were low, as observed by others[19, 20]. By modifying the virus production protocol we could increase the γ-retroviral titers by two logs. The modifications included (I) optimizing the plasmid concentration utilized for virus production (II) shifting from the calcium phosphate method to a transfection reagent and (III) concentrating the virus supernatant (chapter 1). Another crucial aspect to develop in this thesis was the detection of the p47^{phox} transgene expression. p47^{phox} is located within the cytoplasm. In previous p47^{phox} studies, the expression was mainly detected by western blot analyses of crude cell extracts[21] or indirectly by performing functional NADPH oxidase activity assays[18]. This was not suitable for our purposes, as we wanted to analyze the p47^{phox} transgene expression in different hematopoietic cell populations to validate for myelospecific expression. We therefore established the ICS with an anti-p47^{phox} antibody followed by FACS analysis. This allowed us to measure the p47^{phox} transgene expression in the performed second screen and the *in vivo* experiment in GT treated p47^{phox} -/- mice. ICS of the cytoplasmic p47^{phox} transgene product interferes, however, with respiratory burst activity. As the induction of phagocytic NADPH-oxidase activity requires cellular integrity, it is impossible to combine functional analyses with the detection of p47^{phox} protein by immunostaining. To overcome this limitation we developed a system in which the expression of the cytoplasmic transgene product is linked to, and can be monitored by, the co-expression of a cellular surface marker. A single ORF was created encoding the therapeutic transgene linked to the surface marker protein via the FMDV 2A peptide. In this 2A-mediated co-expression system the detection of the surface marker (ΔLNGFR) provides a measure for the expression of the cytoplasmic transgene product (chapter 3). Thereby we could show that the NADPH-oxidase activity was restored in transgenic mouse granulocytes and human macrophages.

The establishment of p47^{phox} ICS enabled us to compare p47^{phox} expression in murine stem- and progenitor cells with the expression in granulocytes in our screen (chapter 1). By far the strongest induction of p47^{phox} expression was observed for the miRNA-223 promoter in our second screen. It is not clear at this stage, why only the miRNA-223 promoter out of all myelospecific promoter candidates showed clear myelospecific induction upon granulocytic differentiation. For all vectors tested, a relatively high p47^{phox} signal in undifferentiated ScaI+ cells was observed. As already mentioned in chapter 1, this is likely due to the presence of unintegrated circular DNA[22] in the stem- and progenitor cells, which is afterwards diluted out upon cell division during granulocytic differentiation. This challenge is hard to circumvent as

analysis after prolonged culture of undifferentiated ScaI+ cells bears the risk of loosing the undifferentiated cells phenotype due to continuous differentiation. If this is true, minor myelospecific expression effects can not be detected by this screen. The stem- and progenitor cells were pre-cultured in media supplemented with different cytokines. Minor changes in the utilized cytokine combination might help to keep the stem- and progenitor cells longer in *ex vivo* culture.

In our screen in p47^{phox} -/- cells, we observed a strong induction of p47^{phox} expression upon myelospecific differentiation by the miRNA-223 promoter, which made this promoter to a promising candidate for an *in vivo* GT in p47^{phox} -/- mice (chapter 2). As expected, the *in vivo* experiment confirmed the myelospecific p47^{phox} expression pattern of the miRNA-223 vector in comparison to the constitutively active SFFV control vector. Furthermore, the miRNA-223 promoter turned out to be highly specific for phagocytes, since there was nearly no or low expression in B- and T-lymphocytes, in stem- and progenitor cells, a weak expression in splenic monocytes, and a strong expression in granulocytes. These results are in line with a performed GT in X-CGD mice in Frankfurt (C. Brendel and M Grez, personal communication), in which the miRNA-223 promoter driving gp91^{phox} expression was utilized as well.

As gene transfer vehicles we have decided to use γ-retroviral SIN vectors. Another possibility would have been the use of a HIV-1-derived lentiviral SIN vectors. Both vector systems show efficient transgene expression levels in hematopoietic cells[23]. In the CGD field γ-retroviral (non SIN) MLV vectors have been successfully used for a long time, even in a clinical study[1]. Up to now there is still a lot more reliable experience in safe production and use of γ-retroviral vectors for clinical GT. Recently it was reported, that even a new PG13-based packaging cell line for stable production of clinical-grade SIN γ-retroviral vectors is available now [24]. We decided therefore to perform our study with γ-retroviral vectors.

Lentiviral vectors have been successfully utilized for the correction of CGD in tissue culture and animal models as well [25, 26]. Roesler and coworkers reported on the correction of the NADPH oxidase activity in granulocytic cells, which were *ex vivo* generated from transduced human CD34+ cells. In the same study, the correction of the CGD phenotype was also reported *in vivo* after transplantation of transduced human HSC into non-obese diabetic (NOD)/SCID mice. A big advantage of the lentiviral vector system is the reduction in time necessary for *ex vivo* manipulation of HSCs. The main reason for this is that lentiviral vectors can enter quiescent cells. An extensive pre-culture with cytokines to bring the stem cells into cell cycle is not required. However, a very important issue for lentiviral vectors is the biosafety considering the pathogenicity of the parental HIV-1 virus in contrast to the MLV virus. Compared to the MLV genome, the HIV-1 genome encodes several accessory proteins

that enhance efficiency of virus life cycle events and are unfortunately associated with the severe pathology of HIV-1. In the past, the design of HIV-1 derived vectors with no ability of generating replication-competent retroviruses (RCR) has been achieved by stepwise changes in vector and packaging elements, which resulted in third "generations" of HIV-1 derived vectors. Third generation terms the safest HIV vector generation up to now[27-29]. Recently, a lentiviral SIN vector was successfully used in clinics to treat 3 patients with cerebral X-linked adrenoleukodystrophy[30]. This study was the first published demonstration of a GT in HSC with a lentiviral SIN vector.

GT vectors based on γ-retroviruses and lentiviruses integrate into the genome of transduced cells with different integration site preferences[31, 32]. MLV was found to integrate preferentially near the start of transcriptional units, whereas lentiviruses prefer integration into coding regions of transcriptional unit, downstream of the transcriptional start site. Whether the difference in integration patterns of MLV or lentiviruses influences the risk of insertional mutagenesis is a matter of debate. On one hand, Naldini and coworkers have found the MLV integration pattern to be significantly more genotoxic than the lentiviral integration pattern in *in vivo* tumor-prone mouse models[17]. On the other hand, in a canine model retroviral integrants of both γ-retrovirus and lentivirus were found within 50kbp around the transcription start site[33]. Considering that activation of proto-oncogene by retroviral integrants can occur over long distance (up to 100kb) the different integration pattern between these two vectors seem to be too minor to influence genotoxicity[8]. In the mentioned lentiviral clinical study for cerebral X-linked adrenoleukodystrophy[30], extensive integration analysis revealed an integration site distribution in gene coding regions as expected for lentiviruses. 36 months after GT, no signs of clonal dominance or even premalignant disproportional distribution of cellular contributions was observed.

The measured $p47^{phox}$ transgene expression in hematopoietic stem- and progenitor cells after GT was markedly reduced with the myelospecific miRNA-223 promoter (in comparison to the constitutive SFFV control), but not suppressed completely. A complementary strategy to target transgene expression to a particular cell type, e.g. granulocytes, could be provided by miRNA regulation[34]. The insertion of a sequence into the untranslated region (3' UTR), which is perfectly complementary to a specific miRNA (which is only present in certain cell populations) restricts the transgene expression to cells which do not express the targeted miRNA. In cells which express the targeted miRNA, the transgene expression is suppressed by degradation of the formed double-stranded RNA. In a recent study several miRNA target sequences were identified and tested in a functional reporter assay[35]. E.g. the miR-126 target site added to the 3'UTR of the transgene was able to inhibit the transgene expression in HSCs and progenitors by endogenous miR-126, but did not prevent the expression in the

differentiated progeny. Therefore inclusion of miR-126 target sites in 3' UTR downstream of the p47^{phox} ORF in addition to the miRNA-223 promoter is likely to suppress transgene p47^{phox} expression in hematopoietic stem- and progenitor cells.

In our X-CGD GT study[1] an increasing discrepancy between gene marking and transgene activity was detected in both patients starting day +320. The gp91^{phox} transgene expression was silenced but the transactivational activity of the viral enhancer persisted, resulting in non-malignant clonal dominance within granulopoiesis[6, 7]. The mammalian chromosome arms contains active chromatin (euchromatin) interspersed with repressive chromatin (heterochromatin). Within these regions the histone modifications play a central role in regulating gene expression. From yeast organisms it is known that the heterochromatic state spreads *in cis* to flanking chromatin by consecutive methylation of adjacent nucleosomes by binding of deacetylating proteins and histone deacetylation[36-38]. Therefore integration of viral construct into silent heterochromatin is expected to result in a spreading of the silent chromatin state into the newly introduced viral sequence, as long as spreading from both sides is not prevented by insulators[39]. One option to prevent transgene silencing caused by spreading histone modifications *in cis* from flanking chromatin is the incorporation of insulator elements flanking the transgene on both sides. A well characterized insulator is the 5 'HSE chicken beta globin insulator (cHS4). Experimentally it could be shown, that the cHS4 insulator possesses enhancer blocking activity and reduces position effects[40]. Silencing can also be initiated from the inside of a newly integrated vector after viral integration into euchromatin. Neither is the recognition machinery for the „foreign DNA" known, nor is the mechanism of retroviral silencing fully understood. Nevertheless, several silencer elements within retroviruses were discovered[41]. In a retrovirus based construct the CHS4 insulator flanking the GFP transgene on both sides failed to block GFP silencing in highly silencing F9 cells[42]. To prevent this form of silencing, the newly integrated and insulated expression unit has to be kept actively in an open chromatin conformation by including a chromatin opening element, e.g. by incorporation of Ubiquitously-acting chromatin opening element (UCOE)[43]. There are indications, that tissue-specific promoters are less prone to silencing compared to a constitutive active promoter like SFFV[44, 45]. However, since there is no precise prediction for the miRNA-223 promoter available, this promoter has to be tested in DNA methylation assays.

Outlook

We have shown in this study that GT in p47^{phox} -/- mice using our miRNA-223 vector resulted in a strictly myelospecific p47^{phox} transgene expression. Furthermore, GT with our vector in p47^{phox} -/- mice functionally corrected the CGD phenotype.

A detailed pre-clinical study is the next step towards a clinical study in human p47^{phox} -/- deficient patients. One important issue is that it has to be shown that the newly developed GT vector is not genotoxic. This has to be shown in serial transplantation of transduced BM stem cells after transduction with high MOI. A malignant transformation in primary or secondary transplanted mice would clearly indicate genotoxic potential.

Furthermore, *in vivo* efficacy of our vector has to be shown in isolated human p47^{phox} -/- BM cells. These cells can be differentiated to neutrophils *ex vivo*, followed by transgene detection and functional analysis of respiratory burst activity[26]. To ensure that the engraftment potential of transduced human HSC is not affected by the vector, human p47^{phox} -/- BM cells can be transduced and transplanted in NOD-SCID mice[26]. As in p47^{phox} -/- mice, p47^{phox} expression can be measured by FACS analysis and reconstitution of superoxide production can be assayed in gene corrected human neutrophils by a DHR assay. This study in addition to the mentioned preclinical experiments should provide more information on longterm myelospecific transgene expression and *in vivo* correction of NADPH oxidase for p47^{phox} deficient form of CGD and in the end should lead to a safer GT based treatment option for p47^{phox} -/- CGD patients.

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Abbreviations

ADA-SCID	adenosine deaminase
AML	acute myeloid leukemia
BM	bone marrow
BMT	bone marrow transplantation
C. albicans	*Candida albicans*
CBP	CREB-binding protein
c/EBPα	C/AAT enhancer binding protein
CGD	chronic granulomatous disease
cHS4	5' HSE chicken beta globin insulator
CIS	common integration site
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRF	coding reading frame
DHR	dihydrorhodamine
ΔLNGFR	truncated human low-affinity nerve growth factor receptor
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dsDNA	double-stranded DNA
E.coli	*Escherichia coli*
EBV	Epstein-Barr virus
EDN	*ex vivo* differentiated neutrophils
EF1α	elongation factor-1 alpha
env	envelope gene
ER	endoplasmic reticulum
ErP	erythrocyte precursor
Ets-1	erythroblastosis virus E26 oncogene homologue 1
Evi1	ecotropic viral integration 1 site
FACS	flow cytometry analysis
FAD	flavin adenin dinucleotide
FBS	foetal bovine serum
FMDV	foot-and-mouth disease virus
gag	group-antigen specific gene
GALV	gibbon ape leukemia virus envelope
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GFP	green fluorescent protein

γ-retroviral	gammaretroviral
GT	gene therapy
H_2O_2	hydrogen peroxide
HBP1	HMG-box containing protein 1
HBSS	Hanks' Buffered Saline Solution
HEK	human embryonic kidney
HLA	human leukocyte antigen
HMG	high mobility group
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
ICS	intracellular staining
IFNγ	interferon-gamma
IN	integrase
IL	interleukin
IRES	internal ribosome entry sequence
IRF-1	interferon regulatory factor 1
ISRE	interferon-stimulated responsive elements
Lin- BM cells	lineage negative bone marrow cells
Lin^- cells	cells which do not express any lineage specific antigens
IVF	*in vitro* fertilization
LAM-PCR	linear amplification-mediated PCR
LCR	locus control regions
LSK cells	Lin-Sca-1+c-kit+ cells
LT-HSC	longterm self-renewing HSC
LTR	long terminal repeat
MDS	myelodysplastic syndrome
MEP	megakaryocyte/erythrocyte progenitors
MFI	mean fluorescence intensity
mG-CSF	murine G-CSF
miRNA-223	microRNA-223
MLV	mouse leukemia virus
MOI	multiplicity of infection
MPP	multipotent progenitor
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NET	neutrophil extracellular traps
NIH	National Institutes of Health

NK	natural killer cells
NOD	non-obese diabetic
OH^-	hydroxyl radicals
ORF	open reading frame
PBS	primer binding site
PCR	polymerase chain reaction
PEA3	polyoma virus enhancer activator 3
PEBP2	phosphatidylethanolamine binding protein 2
PHOX	phagocyte oxidase
PIC	preintegration complex
PMA	phorbol 12-myristate 13-acetate
pol	polymerase gene
polyA	polyadenylation site
PPT	polypurine-tract
PR	viral protease
pro	protease gene
PU.1	Purine Rich Box-1
q-PCR	quantitative PCR
RCR	replication-competent retroviruses
RhoGDI	Rho GDP-dissociation inhibitor
RIS	retroviral integration sites
ROS	reactive oxygen species
RT	enzyme reverse transcriptase
SAEs	serious adverse events
ScaI	stem cell associated antigen
S. aureus	*Staphylococcus aureus*
SCID	severe combined immunodeficiency
SFFV	spleen focus-forming virus
short-term HSC	transiently selfrenewing HSC
SIN	self-inactivating
ssRNA	single strand RNA
TF	transcription factor
UCOE	ubiquitously-acting chromatin opening element
VSV	vesicular stomatitis virus
WPRE	woodchuck hepatitis virus post transcriptional regulatory element
X-CGD	X-linked chronic granulomatous disease

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